Maternal immunity to Newcastle disease in egg yolk of layers

By

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Dedication

To my family,

my father, mother, sisters and brothers.

To my husband,

my sons and my lovely daughter.

To all persons who love me.

Acknowledgment

First of all I thank the greatest Allah for his generosity and prosperity.

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Finally, I am thankful to my family for the greatest gift I could ever receive, the touch of care and sense of love.

Abbreviations

Ab	Antibody
Ag	Antigen
APMV-1	Avian Paramyxovirus serotype one
APMV-9	Avian Paramyxovirus serotype nine
ELISA	Enzyme linked immunosorbent assay
H_2O_2	Hydrogen peroxide
ICPI	Intracerebral pathogenicity index
Kb	Kilobase
mg	Milligram
min	Minute(s)
ml	Milliliter
ND	Newcastle disease
NDV	Newcastle disease virus
nm	Nanometers
PMV-1	Paramyxovirus serotype one
RNA	Ribonucleic acid
V4-HR	Heat resistant strain of V4 Newcastle disease virus vaccine
µl	Microliter

Abstract

This study was carried out on ninety six egg samples collected from laying hens from commercial chicken farm at Alhalfaia (Khartoum North). The hens were vaccinated against Newcastle disease (ND) with the Newcastle disease virus (NDV) vaccine (Komorov strain) produced in the Central Veterinary Research Laboratories center (Khartoum, Sudan).This experiment attempt to detect the presence of immunoglobulin Y (IgY) in the egg yolk as a result of transfer of maternal antibodies from immunized hen's sera to the egg yolk and to study the specificity of these antibodies to NDV.The immunoglobulin Y(IgY) was extracted from egg yolk by dextran sulphate method. The method of extraction consists of three steps: Precipitation of lipids by dextran sulphate; precipitation of proteins by sodium sulphate; and dialysis of precipitate against tris- buffered saline (TBS).

The presence of IgY in egg yolk, antibody titers and specificity to Newcastle disease virus (NDV) were determined by standardized indirect ELISA (In- house ELISA). All Ninety six samples were found positive (100%). The positive results were varied in titers between $\log_2$ 13.643 (the maximum) and $\log_2$ 8.643 (the minimum). As comparative study, detection of maternal specific NDV IgY was done using NDV antibody test kit. All samples tested were found positive and antibody titers were varied between $\log_2$ 14.171 (the maximum) and $\log_2$ 10.838 (the minimum). It was found that titers obtained from NDV antibody test kit were higher than those obtained from the standardized indirect ELISA. From this study, it was found that maternally derived IgY antibodies present in the egg yolk specific to NDV were present in high titers that confer protection during early weeks of life of newly hatched chicks. Extraction of maternally derived specific NDV antibodies from yolk will facilitate accurate monitoring of ND vaccination programmes.

ملخص الأطروحه

اجريت هذه الدراسه على ست و تسعون عينة بيض جمعت من دجاج بياض من احدى مزارع الدواجن التجاريه بمنطقة الحلفايا شمال الخرطوم. هذا الدجاج تم تطعيمه ضد مرض النيوكاسل (مرض سمير) بلقاح مرض النيوكاسل، عترة الكموروف المنتج في مركزالمعامل والبحوث البيطريه المركزية (الخرطوم، السودان). هذه التجربه هي محاوله للكشف عن وجود الاجسام المضاده نوع IgY في مح البيض والموجوده كنتيجه لانتقال المناعه الاميه من مصل الامات الممنعه الى مح البيض ومن ثم دراسة خصوصية هذه الاجسام المضاده لفيروس مرض النيوكاسل. تم استخلاص الاجسام المضاده IgY من مح البيض بطريقة كبريتات الدكستران. تتكون هذه الطريقه من ثلاث مراحل: ترسيب الدهون بواسطة كبريتات الدكستران، ترسيب البروتين بواسطة كبريتات الصوديوم ثم الديلزة. حدد وجود الاجسام المضادهIgY ، معيارها وخصوصيتها لفيروس مرض النيوكاسل بواسطة الاليزا غير المباشره المحضره بالمعمل. وجدت جميع العينات موجبة لوجود الاجسام المضاده في مح البيض(100%). تراوح معيار الاجسام المضاده في العينات الموجبة بين اعلى معيار13.643 واقل معيار8.643. للمقارنة تم الكشف عن وجود الاجسام المضاده لفيروس مرض النيوكاسل باستعمال طقم الاليزا. وجدت جميع العينات موجبة لوجود الاجسام المضاده في مح البيض (100%). تراوح معيار الاجسام المضاده في العينات الموجبة بين اعلى معيار 14.171 وأقل معيار10.838 كما وجد أن معيار الاجسام المضاده المتحصل عليه من هذه التجربه أعلى من ذلك المتحصل عليه من تجربة الإليزا غير المباشرة المحضره بالمعمل.

من هذه الدراسة وجد ان الاجسام المضاده المنحدره من اصل امي والموجوده في مح البيض لها خصوصيه لفيروس مرض النيوكاسل وتتواجد بمعاييرعالية تمنح الوقاية للكتاكيت ضد مرض النيوكاسل خلال الأسابيع الأولى بعد الفقس. استخلاص الأجسام المضاده لفيروس مرض النيوكاسل والمنحدره من اصل امي من مح البيض يساعد في تقييم برامج التلقيح ضد مرض النيوكاسل.

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INTRODUCTION

Very young chicks are susceptible to many pathogens during the first few weeks of age because their immune system is not fully developed (*Hamal et al*., 2006). An evolutionary attempt to compensate for the immaturity is expressed in a maternal immunity component consisting of antibody absorbed from the egg and provided by the dam in a proportionate manner (*Ask et al*., 2004). Hence, maternal antibodies are the primary means of antigen-specific protection (*Hamal et al*., 2006).

Chickens are susceptible to many infectious diseases. One of the most important of these is the viral disease known as Newcastle disease, which causes devastating losses in both commercial and village chickens (*Grimes*, 2002). ND is caused by avian paramyxovirus serotype one (APMV–1) viruses (*Aldous* and *Alexander*, 2001). This disease can be controlled by the use of vaccines and there are many Newcastle disease vaccines suitable for use in chickens (*Grimes*, 2002). Because of the severe nature of the disease and the associated consequences, ND is included as an Office Internationale des Epizooties (OIE) list a disease (*Office Internationale des Epizooties*, 2001). Nevertheless, ND is enzootic in some areas of the world and remains a constant threat to most birds (*Aldous* and *Alexander*, 2001). Chickens that survive infection with virulent Newcastle disease virus develop a long lasting immunity to further infection with Newcastle disease virus. The basis of this immunity is circulating antibodies, secretory antibody (producing mucosal immunity) and cell mediated immunity (*Grimes*, 2002). Hens with antibodies to NDV will pass these on to their progeny via the egg yolk. The populations of IgY are transported according to their concentration in the maternal serum (*Carlander*, 2002) and thus levels of antibody in day- old chicks will be directly related to titers in the parent (*Alexander*, 2003). Maternal antibodies

protect chicks for three to four weeks after hatching (*Murphy et al*., 1999). There is no doubt that maternal antibody can influence the response to vaccination during the first weeks of life (*McMullin*, 1985).

Seromonitoring of humoral immune response in vaccinated chicken flocks is necessary for controlling Newcastle disease. In regular antibody detection, bleeding of animal is necessary to purify antibodies from the blood serum. Bleeding of chickens is considered painful and usually causes large haematoma because chickens have fragile veins. Chicken egg yolk IgY offers an easy and acceptable alternative for production of antiserum.

Several methods can be used for the extraction of IgY from egg yolk, and commercial extraction kits are available (*Schade et al*., 1996). The choice of method is a matter of yield and purity desired, final use of the IgY as well as material cost and labor skills (*Carlander*, 2002). A number of methods have been published for the detection of antibodies against Newcastle disease virus (NDV) by means of enzyme-linked immunosorbent assays (ELISAs) and commercial kits have also been produced (*Bell*, *et al*., 1991).

The main aims of the present study were i) to extract IgY antibodies from egg yolk ii) to elaborate in-house ELISA for measurement of specific maternally derived NDV antibodies and iii) to test the specificity of egg yolk antibodies to Newcastle disease virus.

CHAPTER ONE
LITERATURE REVIEW

1.1 Definition

Newcastle disease (ND) is a deadly viral disease of poultry causing high mortality due to its high contagiousness and rapid spreading among chicken and other domestic and semi-domestic species of birds (*Rahman et al*., 2002 and *Murphy et al*., 1999). The virus has a wide host range, most orders of birds reported to have been infected by Newcastle disease virus (NDV) (*Seal et al*., 2000). It is enzootic (endemic) in most countries in Africa, Asia and South America, where it continues to cause serious losses despite the vaccination of industrialized poultry (*Tabidi et al*., 2004).

The 'OIE' defined ND as "a disease of birds caused by strains of Avian PMV-1, significantly more virulent than lentogenic strain..." whereas the European Council Directives defined ND as "an infection of poultry caused by Avian PMV-1 in day-old chicks with ICPI greater than >0.7."(*Survashe* and *Desmukh*, 1998).

1.2 Disease history

Newcastle disease has been one of the most important diseases of poultry worldwide. The disease was first observed in Java in 1926 (*Murphy et al*., 1999 and *Seal et al*., 2000), and in the same year it spread to England, where it was first recognized in Newcastle, hence the name Newcastle disease. (*Murphy et al*., 1999 and *Alexander*, 1988). The agent of ND was recovered in 1926 by Doyle from diseased birds (*Hofsade et al*., 1978).

1.3 Classification of Newcastle disease virus (NDV)

Newcastle disease virus is a member of the *Paramyxoviridae* family (*Seal et al*., 2000) in the order *Mononegavirales*. This virus family is divided into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*. The

subfamily *Paramyxovirinae* has three genera: *Rubulavirus*, *Respirovirus* and *Morbillivirus* (*Alexander*, 2003). In 1993 the International Committee on Taxonomy of Viruses rearranged the order of the Paramyxovirus genus and placed NDV within the *Rubulavirus* genus among the *Paramyxovirinae* (*Seal et al*., 2000).

Nine serogroups of avian paramyxoviruses have been recognized, APMV-1 to APMV-9 (*Alexander*, 2003). ND is caused by avian paramyxovirus serotype 1 (APMV–1) viruses. Recent work involving the sequencing of the whole NDV genome has suggested that avian paramyxoviruses are sufficiently different from other rubulaviruses to warrant placing them in a separate genus (*Aldous* and *Alexander*, 2001).

1.4 Virus structure

1.4.1 The virion

Paramyxovirus virion is pleomorphic in shape (spherical and filamentous forms occur), 150-300nm in diameter. A virion is enveloped, covered with large peplomers (8-20 nm in length), and contain "herringbone-shape" helically symmetrical nucleocapsid, 600-800 nm in length and 18 nm in diameter. The spikes (peplomers) shown in *Figure* (1) are composed of two glycoproteins: a haemagglutinin- neuraminidase (HN) protein and fusion (F) protein, which are the antigenic components that stimulate the host to produce haemagglutinin - inhibiting and virus – neutralizing antibodies (*Hofsade et al*., 1978 and *Murphy et al*., 1999)

1.4.2 Newcastle disease virus genome

The virus genome consists of a single linear molecule of negative- sense, single- stranded RNA, approximately 15-16 kb in size (*Murphy et al*., 1999), which codes for six proteins including RNA directed RNA polymerase (L), haemagglutinin- neuraminidase (HN) protein, fusion (F) protein, matrix (M)

protein, phospho-protein (P) and nucleoprotein (NP) as shown in *Figure* (2). Transcription occurs in the 3' to 5' direction (*Seal et al*., 2000). The HN glycoprotein is responsible for virus attachment to the cell surface receptors. The F glycoprotein is responsible for fusion between the cellular and viral membranes and subsequent virus genome penetration (*Aldous* and *Alexander*, 2001).

1.5 Virus replication

Intracellular virus replication takes place within cytoplasm. Because the virus RNA has negative sense, the viral RNA-directed RNA- polymerase (transcriptase) must produce complementary transcripts of positive sense that may act as messenger RNA and use the cell's mechanisms, enabling the translation into proteins and virus genomes. The F protein is synthesized as a non functional precursor, F0 that requires cleavage to F1 and F2 by host proteases. This cleavage has significance in the pathogenicity of NDV strains. The HN of some strains of NDV also requires posttranslational cleavage (*Alexander*, 2003).

1.6 Virus properties

1.6.1 Physico-chemical properties

The infectivity of NDV and other avian paramyxoviruses may be destroyed by physical and chemical treatments such as heat, irradiation (including light and ultra violet rays), oxidation processes, pH effects, and various chemical compounds. The rate at which infectivity is destroyed depends on the strain of the virus, the length of exposure, the nature of the suspending medium, and interactions between treatments. No single treatment can guarantee destruction of all viruses but may result in a low probability of remaining infective virus (*Alexander*, 2003).

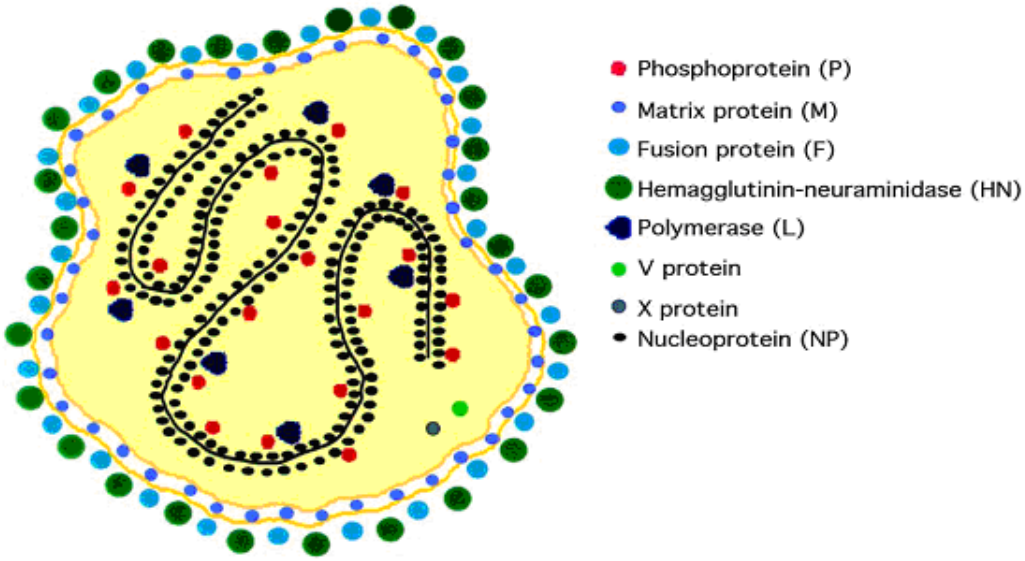


Fig. 1: Newcastle disease virus structure.
Newcastle disease virus (NDV) is an enveloped virus with two surface glycoproteins, the fusion (F) and attachment haemagglutinin-neuraminidase (HN) proteins. The matrix (M) protein is juxtaposed between the envelope and the interior nucleocapsid structure. The nucleoprotein (NP), phosphoprotein (P) and polymerase (L) proteins make up the transcriptase complex and are in close contact with the viral genome (*Seal et al*., 2000).

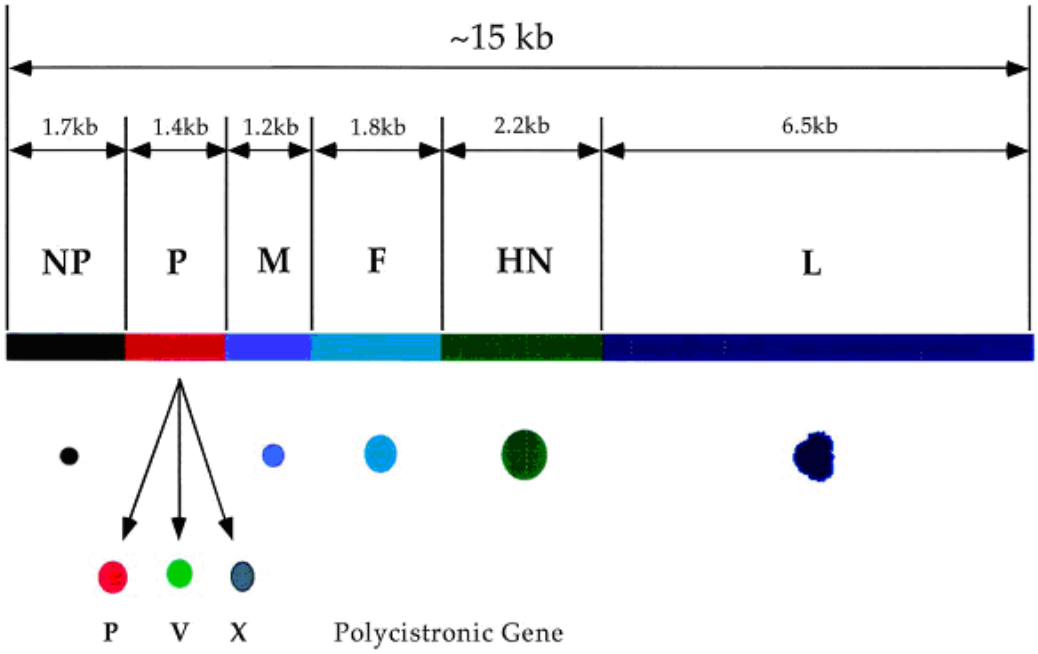


Fig. 2: Newcastle disease virus genomic organization.
NDV genome is a single-strand,negative sense RNA encoding six open reading frames. The P protein gene is polycistronic due to insertion of at least one additional guanosine during transcription and utilization of potential alternative transcription start sites (*Seal et al*., 2000).

1.6.2 Biological properties

1.6.2.1 Haemagglutination

All strains of Newcastle disease virus agglutinate chicken red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemagglutination. Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles (*Grimes*, 2002).

The process of haemagglutination consists of the attachment of the virus to the receptor substance on the cell (erythrocyte) surface causing clumping of the cells (agglutination) followed later by the destruction of the receptor substance by the enzyme neuraminidase. This second step is associated with the release (elution) of the virus from the surface of the cell (*Hofsade et al*., 1978).

Antibody response to the haemagglutinin protein in the Newcastle disease virus envelope can be measured by the Haemagglutination inhibition (HI) test. When serum containing these antibodies is mixed with Newcastle disease virus, the antibodies bind to the haemagglutinin protein in the envelope of the virus. This blocks the haemagglutinin protein from binding with the receptor site on chicken red blood cells. Thus the haemagglutination reaction between the virus and the red blood cells is inhibited (*Grimes*, 2002). Haemagglutination inhibition (HI) test is the most widely used for measurement of antibodies (Abs) against Newcastle disease virus (NDV) (*Tabidi et al*., 2004).

1.6.2.2 Haemolysis

NDV possesses a haemolysin. The virus is capable of lysing those erythrocytes that it can agglutinate (*Hofsade et al*., 1978).

1.7 Epidemiology

Over 250 species of birds have been reported to be susceptible to NDV as a result of natural or experimental infections, and it is likely that many more susceptible species exist but have not yet been identified (*Alexander*, 1997).

1.7.1 The virus-host interaction

Newcastle disease virus reacts with avian hosts in various ways. When non-immune domestic chickens encounter highly pathogenic strains of Newcastle disease virus, the common sequel is an acute disease with mortality close to 100%. The birds will often be paralyzed or have twisted necks. This severe disease, attributed to velogenic strains of Newcastle disease virus, occurs in both village and commercial poultry. In commercial chickens, strains of Newcastle disease virus of moderate virulence (mesogenic strains) cause lower rates of mortality in mature chickens, but severely deplete egg production. Strains of low virulence (lentogenic strains) cause little mortality except in young birds, but do reduce egg production. These strains also interact synergistically with other pathogens, especially respiratory pathogens, to produce severe clinical disease (*Spradbrow*, 1990).

1.7.2Transmission

Transmission occurs by direct contact between birds by the airborne route via aerosols and dust particles and via contaminated feed and water. With lentogenic strains, transovarial transmission is important and virus-infected chicks may hatch from virus-containing eggs. Trade in infected avian species and products plays a key role in the spread of Newcastle disease from infected to noninfected areas. Virus may also be disseminated by frozen chickens, foodstuff, bedding, manure, and transport containers. In birds that survive, the virus is shed in all secretions and excretions for at least four weeks (*Murphy et al*., 1999).

1.8 Pathogenicity

The molecular basis for NDV pathogenicity is dependent on the F protein cleavage site amino acid sequence and the ability of specific cellular proteases to cleave the F protein of different pathotypes (*Seal et al*., 2000). Strains of Newcastle disease virus differ widely in virulence, depending on the cleavability and activation of their hemagglutinin and fusion glycoprotein (*Murphy et al*., 1999). The term velogenic (viscerotropic), mesogenic, and lentogenic are applied to Newcastle disease virus strains of high, intermediate, and low virulence respectively (*Murphy et al*., 1999 and *Seal et al*., 2000). Avirulent strains of Newcastle disease virus causes no disease (*Grimes*, 2002). Whereas velogenic strains kill virtually 100% of infected fowl, naturally avirulent strains have even been used as vaccines (*Murphy et al*., 1999).

Initially the virus replicates in the mucosal epithelium of the upper respiratory and intestinal tracts; shortly after infection, virus spreads via the blood to the spleen and bone marrow, producing a secondary viremia. This leads to infection of other target organs: lung, intestine, and central nervous system (*Murphy et al*., 1999).

1.8.1Clinical features

In chickens, respirator, circulator, gastrointestinal, and nervous signs are seen; the particular set of clinical manifestations depends on the age and immune status of the host and on the virulence and tropism of the infecting strain (*Murphy et al*., 1999). The incubation period of ND after natural exposure has been reported to vary from 2-12 days and an average five to six days (*Alexander*, 2003).

A combination of inspiratory dyspnea (gasping), cyanosis of comb and wattles, and clonic muscular spasm is indicative. There is a loss of appetite, listlessness; Intestinal symptoms may include crop dilatation, presence of

foamy mucus fibrinous exudate in the pharynx, a similar discharge from the beak, and yellow-green diarrhea. Nervous system involvement is indicated by paralysis of wings and/or legs, torticollis, ataxia or circular movements, bobbing-and-weaving movements of the head, and clonic spasms. In layers there is a sudden decrease in egg production together with depigmentation and/or loss of shell and reduction in the albumen quality of eggs (*Murphy et al*., 1999).

1.8.2Pathology

Gross pathologic findings include ecchymotic haemorrhages in the larynx, trachea, esophagus, and throughout the intestine. The most prominent histologic lesions are necrotic foci in the intestinal mucosa and the lymphatic tissue and hyperemic changes in most organs, including the brain (*Murphy et al*., 1999).

1.8.3 Human disease

Newcastle disease virus can produce a transitory conjunctivitis in humans; usually mild and persisting one to two days but on occasion quite sever and even leading to some lasting impairment of vision. The condition has been seen primarily in laboratory workers and vaccination teams exposed to large quantities of virus. The disease has not been reported in individuals who rear poultry or consume poultry products (*Murphy et al*., 1999 and *Hofsade et al*., 1978).

1.9 Immunity to Newcastle disease virus

Chickens that survive infection with virulent Newcastle disease virus develop a long lasting immunity to further infection with Newcastle disease virus. The basis of this immunity is circulating antibodies, secretory antibody (producing mucosal immunity) and cell mediated immunity. Newcastle disease virus of low virulence induces similar immune responses without

causing severe disease. This is the basis of vaccination (*Grimes*, 2002). A summary of antiviral defence mechanisms is illustrated in *Figure*3.

1.9.1 Cell-mediated immune response

Cell-mediated immunity is the initial immune response to infection with NDV and may be detectable as early as two to three days after infection with live vaccine strains. This has been thought to explain the early protection against challenge that has been recorded in vaccinated birds before measurable antibody response is seen. The cell- mediated immune response to NDV by it self is not protective against challenge with virulent NDV (*Alexander*, 2003).

1.9.2 Humoral immune response

Antibody production is rapid (*Murphy et al*., 1999). Antibody directed against either of the functional surface glycopolypeptides, the HN and the F polypeptides can neutralize NDVs (*Alexander*,2003) Haemagglutination-inhibiting antibody can be detected within four to six days of infection and persists for at least two years (*Murphy et al*., 1999). Peak response is at about three to four weeks (*Alexander*, 2003). The level of Haemagglutination-inhibiting antibody is a measure of immunity. IgG is confined to the circulation and does not prevent respiratory infection, but it does block viremia; locally produced IgA antibodies play an important role in protection in both the respiratory tract and the intestine (*Murphy et al*., 1999).

1.9.2.1 Immuoglobulins in chicken

Three immunoglobulin classes have been shown to exist in chicken, IgA, IgM and IgY (IgG) (*Carlander*, 2002). IgM appears after four to five days following exposure to a disease organism and then disappears by 10-12 days. IgG (IgY) is detected after five days following exposure, peaks at three to three and a half weeks, and then slowly decreases. IgA appears after five days following exposure.

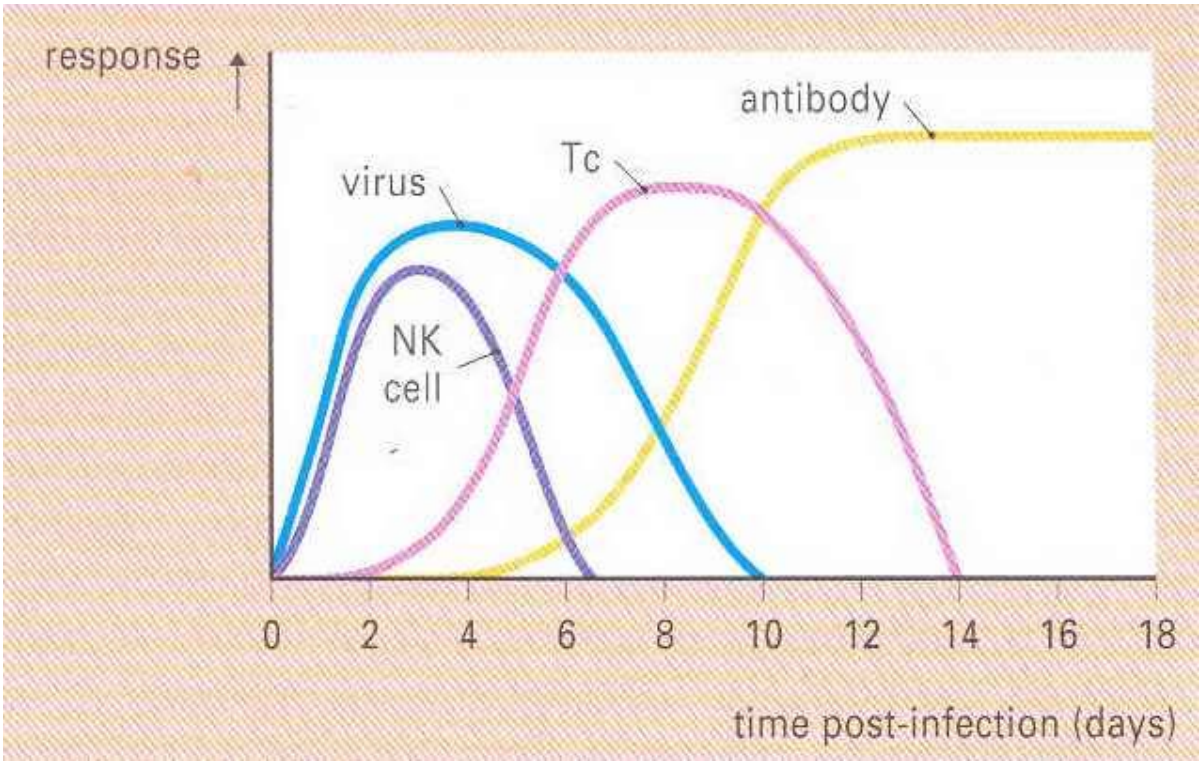


Fig. 3: Kinetics of host defenses in response to a typical acute virus infection. Natural killer (NK) cells and interferon (IFN) are detected in the blood stream and locally in infected tissues.Cytotoxic T cells (Tc) then become activated followed by the appearance of neutralizing antibodies in serum (*Roitt et al*., 2001).

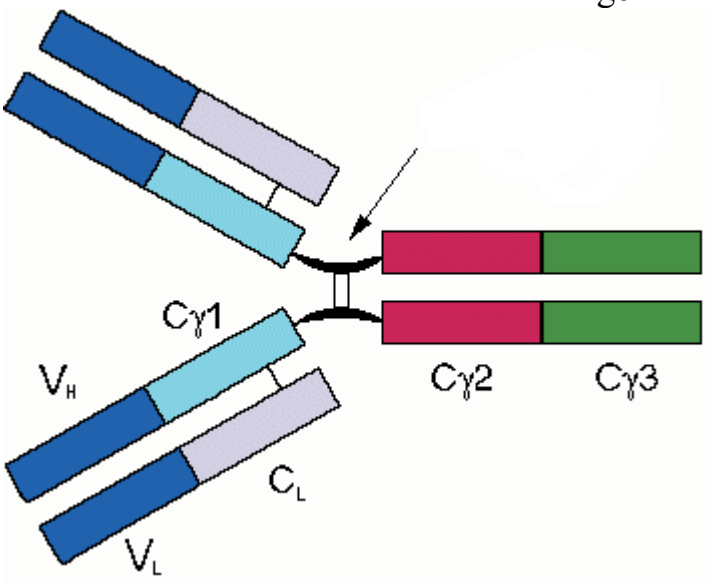
This antibody is found primarily in the mucus secretions of the eyes, gut, and respiratory tract and provides "local" protection to these tissues (*Butcher* and *Miles*, 2003).

1.9.2.2 Chicken IgY

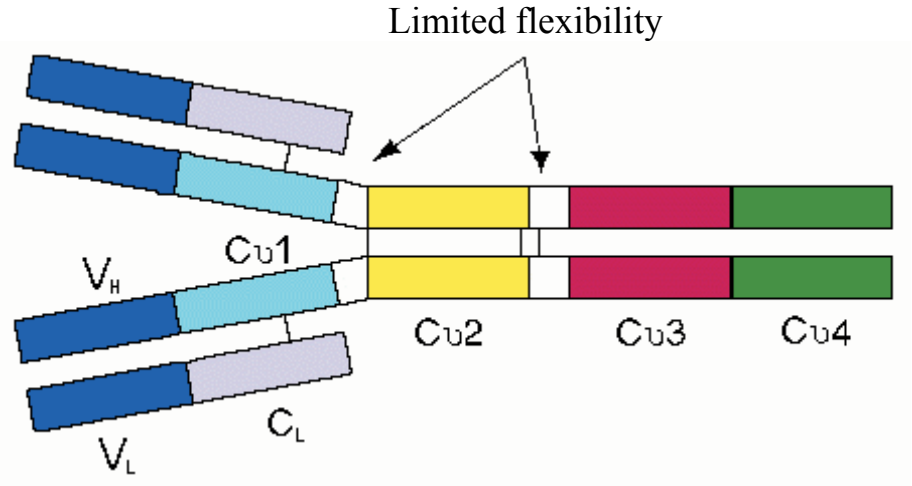
The terms IgG and IgY are commonly interchanged when speaking of chicken immunoglobulin. Immunoglobulin from chickens and other avian species bear some resemblance to mammalian IgG, but also display some unique structural and functional characteristics that distinguish them from IgG (*Stone et al*., 1992). General structure of IgY molecule is the same as of IgG with two heavy chains (HC) with a molecular mass of 67–70 kDa each and two light chains (LC) with a molecular mass of 25 kDa each. The major difference is the number of constant regions (C) in heavy chains: IgG has three C regions, while IgY has four C regions. One additional C region with two corresponding carbohydrate chains has a logical consequence in a greater molecular mass of IgY compared to IgG i.e. 180 and 150 kDa, respectively. Narat (2003) reported that IgY is much less flexible than IgG due to the absence of the hinge between C1 and C2, which is a unique mammalian feature (*Figure* 4).

IgY is the accepted/proper term for chicken antibodies. Chicken IgY is the functional equivalent to mammalian IgG. It is found in the serum of chickens and passed from the mother chicken to the embryo via the egg yolk, imparting a high concentration of chicken IgY to developing embryo. The "Y" in IgY comes from "yolk" and is the main antibody component in the egg yolk (*Stone et al.*, 1992).

Chicken IgY is a systemic rather than a secretory antibody but IgY is also found in duodenal contents, tracheal washings and seminal plasma. It is called



Mammalian IgG



Avian IgY

Fig.4: Structure of mammalian IgG and avian IgY.
The major difference is the number of constant regions (C) in heavy chains: IgG has three C regions, while IgY has four C regions. IgY is much less flexible than IgG due to the absence of the hinge between C1 and C2 (*Narat*, 2003).

IgY rather than IgG to distinguish it from its mammalian counterpart (*Carlander*, 2002).

In chickens, it has been well established that IgG (IgY) is the antibody isotype that is transferred from the dam to her offspring (*Hamal et al*., 2006) and is the major antibody found in eggs (*Carlander*, 2002). Other classes are present, but only in negligible amounts (*Schade et al*., 1996). In eggs, IgY is present predominantly in the egg yolk, whereas IgA and IgM are present in the egg white (*Hamal et al*., 2006).

1.9.2.3 Stability of chicken IgY

IgY is very stable under normal conditions. IgY antibodies have been stored for over 10 years at 4°C without any significant loss in antibody activity. Chicken antibodies have also retained their activity after six months at room temperature or one month at 37 °C (*Raj et al*., 2004).

IgY is a protein and as such, is sensitive to denaturation. However, IgY is fairly heat stable (*Carlander*, 2002 and *Szabo et al*., 1998), and most antibody activity remain after 15 minutes at 70°C. Incubation of IgY at pH above four is well tolerated, but at pH 2 and 37°C the activity is rapidly decreased (*Carlander*, 2002).

1.9.3 Maternal Immunity

Maternal antibody transmission is defined as the transfer of antibodies by an immunocompetent adult, typically a female, to an immunologically naive neonate transplacentally or through colostrum, milk, yolk, etc. The ability of mothers to transmit antibodies to their offspring was documented in both mammals and birds over 100 years ago (*Grindstaff et al*., 2003).

In many animals immunity is not fully developed until adulthood but the young still need protection against various sets of pathogens. Thus, bird nestlings are highly dependent on antibodies received from their mother

(in the eggs) during their rapid early growth period. The relationship between maternal immunity and the development of neonates' own immunity has been poorly studied (*Pihlaja et al*., 2006).

An evolutionary attempt to compensate for the immaturity is expressed in a maternal immunity component consisting of antibody absorbed from the egg and provided by the dam in a proportionate manner (*Ask et al*., 2004). Hens with antibodies to NDV will pass these on to their progeny via the egg yolk. Levels of antibody in day- old chicks will be directly related to titers in the parent (*Alexander*, 2003). Maternal antibodies protect chicks for three to four weeks after hatching (*Murphy et al*., 1999). The advantages of maternal immunity are that it provides early age protection against pathogens, and that it prevents unfavorable development of tolerance to pathogens. Effects are however controversial, as it can also hinder stimulus and activation of the chick's own immune system (the innate and the acquired immunity). External stimulus is vital for development of this, and a critical stage eventuates when maternal protection fades (two to four weeks of age depending on the initial amount of maternal antibody in the chick) (*Ask et al*., 2004). Very young chicks are susceptible to many pathogens during the first few weeks of age because their immune system is not fully developed; hence, maternal antibodies are the primary means of antigen-specific protection. There are many reports in the literature regarding the transfer of pathogen-specific antibodies from hens to their chicks via the egg and their role in the protection of newly hatched chicks from the pathogens. The time at which the newly hatched chicks start to synthesize antibodies endogenously depends on the type of antibody. Lawrence et al. in 1981cited by (*Hamal et al*., 2006) reported that IgY-secreting B cells are not detectable in a chick's plasma until six days post hatch.

1.9.3.1Transport of IgY from maternal serum to the offspring

It was in 1893 that Klemperer first described the acquisition of passive immunity in birds, by demonstrating the transfer of immunity against tetanus toxin from the hen to the chick (*Carlander*, 2002 and *Schade et al*., 2005). In 1901 Dziergowski (cited by Mahasin E. Abdel-Rahman , M.Sc. thesis,1980, Cambridge, UK) reported the passage of diphtheria antitoxin in unchanged serum immunoglobulin from hens' sera to the growing egg and then from the yolk to the embryo and the chick. Jukes, Fraser and Orr in 1934 reported that there were comparable amounts of antitoxin in the serum and in the livetin of egg after the injection of antitoxin in chicken. In 1946 antibodies to Newcastle disease were first demonstrated by Brandly, Moses and Jungherr (cited by Mahasin E. Abdel-Rahman, M.Sc. thesis, 1980, Cambridge, UK). They showed that these antibodies could be transferred from laying hens to the yolk and thence to the developing embryo and embryonic tissues. Since the 1980s, egg yolk antibodies (IgY Abs) have found a broader application, possibly due to the availability of commercial secondary reagents such as IgY-purification kits, IgY-standards, and of labelled Abs (such as alkaline phosphatase, fluorescein isothiocyanate and peroxidase) specifically against IgY (*Schade et al*., 2005).

Female birds transmit passive immunity to offspring through the deposition of anti-bodies in eggs (*Grindstaff et al*., 2003 and *Hamal et al*., 2006). The transport of IgY from the hen serum to the offspring is a two-step process. First IgY is transported from the serum to the egg yolk in analogy to the cross-placental transfer of antibodies in mammals. The second step is the transmission of IgY from the yolk sac to the developing embryo (*Carlander*, 2002 and *West et al*., 2004).

IgY is taken up into the egg yolk by the IgY receptors on the ovarian follicle from the dam's blood. In the second step, IgY is transferred from the egg yolk to the offspring via the embryonic circulation (*Hamal et al*., 2006). The IgY receptors on the oocyte bind and move all populations of IgY from the hen serum to the egg (*Carlander*, 2002).

The concentration of IgY in the yolk is essentially constant through the oocyte maturation, and at maturity the yolk will contain about 10-20 mg/ml IgY. Looking at the egg, IgY is not present in the egg white while IgA and IgM are not present in the yolk. There is about 100-400 mg IgY packed in the egg. Labeled IgY binds specifically to yolk sac tissue from day seven up to at least day 18. The populations of IgY are transported according to their concentration in the maternal serum. There is no selection nor destruction of IgY during transport and the yolk IgY has the same amount of sialic acid as the serum IgY. The amount of IgY transported is independent of egg size (*Carlander*, 2002) and known to be proportional to the maternal serum IgY concentration (*Hamal et al*., 2006 and *Carlander*, 2002).

In the newly hatched chick the IgY concentration in circulation is about 1-1.5 mg/ml and the circulating half-life of IgY is about 36 hours. IgY secreting cells in the offspring are not detectable until six days after hatching (*Carlander*, 2002).

Structurally, IgY is identical to the major immunoglobulin (Ig) found in serum (*Schade et al*., 1996 and *Szabo et al*., 1998). There is still controversy about the relative concentrations of the different types of Igs found in egg yolk and serum; the data available indicate that IgY is more highly concentrated in yolk than it is in serum (*Schade et al*., 1996 and *Raj et al*., 2004).

IgY levels, total or antigen-specific, in the dams' plasma or eggs were found to be a direct indicator of maternal antibody transfer to the chicks'

circulation, with an expected percentage transfer of approximately 30%. This knowledge, may find direct application in formulating strategies for protecting chicks, especially during the first few weeks of age when their immune system is not yet fully functional (*Hamal et al*., 2006).

1.9.3.2 Maternal immunity and vaccination

Factors which interfere with immunization of commercial poultry can be divided into three main groups. They are: factors associated with the vaccine itself, those of vaccine administration, and those which are endogenous to the bird. Circulating antibody may affect the response to vaccination. Baby chicks at one to three days of age have circulating antibodies in similar concentrations to those found in dams. The titers fall to be undetectable by 14 - 30 days (depending on the method of detection used). There is no doubt that maternal antibody can influence the response to vaccination during the first weeks of life (*McMullin*, 1985). Maternal antibody interferers with active immunization, presumably by sequestering vaccine antigen or restricting replication of vaccine virus (*Stone et al*., 1992). Maternal antibody is protective and thus, taken into consideration during primary vaccination. It was reported that maternal antibody neutralizes the introduced vaccine antigen rendering the vaccine ineffective. It was mentioned that immune response was nil at high titer of maternal antibody (*Rahman et al*., 2002).

1.9.3.3 Simulation of maternal immunity

Simulation of maternal immunity by inoculation of immune yolk preparation in to the yolk sac of one-day-old chickens was done by Stone *et al*. (1992). In their work, yolk was harvested from eggs laid by hens hyperimmunized with killed Newcastle disease virus (NDV) and inoculated into the yolk sac of one-day-old specific-pathogen-free (SPF) chickens. Serum

haemagglutination-inhibition antibody titers reached maximum levels one to four days after yolk inoculation and declined at a rate similar to that reported for naturally acquired maternal antibody. Expected levels of immune interference were observed when yolk-inoculated chickens were vaccinated with a conventional oil-emulsion NDV vaccine. These results show that yolk-sac inoculation with yolk antibody is a suitable approach for producing maternally immune chickens for laboratory studies.

In 1980, the simulation was done also by Mahasin E. Abdel-Rahman. She used the whole yolk from eggs produced from hyperimmunized hens. In her experiment one- day old chicks hatched from non- immune hens were injected subcutaneously with immune yolk. The antibodies were demonstrable in chick's blood two hours after injection. The immunity produced was last only for few weeks. She reported that passive immunization for one-day old chicks is important because one-day old chicks may not respond efficiently to active vaccination while they can readily absorb passively injected antibodies. This may have its implication in areas where Newcastle disease is endemic and protection is needed at all times.

1.10 Laboratory Diagnosis of NDV

Because clinical signs are relatively nonspecific and because the disease is such a threat, diagnosis must be confirmed by virus isolation and serology. Diagnosis can be attempted about one week after onset of the symptoms and repeated one week later in case of inconclusive results (*Murphy et al*., 1999).

1.10.1 Virus isolation

The virus may be isolated from spleen, brain, or lungs by allantoic inoculation of 10-day-old embryonated eggs, with the virus being differentiated from other viruses by haemadsorption- and haemagglutnation-

inhibition tests. Virus can be isolated from the gut when circulating antibodies are already present (*Murphy et al*., 1999).

Pathotype prediction initially involves NDV inoculation of embryonated eggs to determine mean death time of the embryo (MDT). Further testing entails inoculation of chickens to determine the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) (*Seal et al*., 2000).

1.10.2 Serological tests

Immunofluorescence on tracheal sections or smears is rapid although less sensitive. Demonstration of antibody is diagnostic only in unvaccinated flocks. Haemagglutination- inhibition (HI) test is the most widely used for measurement of antibodies (Abs) against Newcastle disease virus (*Tabidi et al*., 2004). The haemagglutnation-inhibition test is also used for surveillance of chronic Newcastle disease in countries where this form of the disease is endemic (*Murphy et al*., 1999).

Enzyme-linked immunosorbent assays (ELISAs) have also been employed for the detection of antibodies against NDV. ELISA technique is more accurate, sensitive and rapid to perform in detecting Abs against NDV compared to HI test although the later is more economic (*Tabidi et al*., 2004).

1.10.3 Molecular techniques in the diagnosis of NDV

The attraction of molecular-based techniques in ND diagnosis is that they may be able to cover all three aspects of Newcastle disease diagnosis (detection of virus, characterization, including inference of virulence, and epidemiology) quickly, accurately and definitively in a single test (*Aldous* and *Alexander*, 2001). The most molecular technique used for the diagnosis of ND is the reverse transcription- polymerase chain reaction (RT-PCR) because NDV has an RNA genome (*Alexander*, 2003), with subsequent analysis of the

product by restriction enzyme analysis, probe hybridization and nucleotide sequencing (*Aldous* and *Alexander*, 2001). Oligonucleotide probes and viral genomic RNA fingerprint analysis have been used to identify and differentiate NDV strains, but with limited success. Monoclonal antibodies are now used to identify antigenic groups (*Seal et al*., 2000).

1.11 Prevention and control

There are national and international policies framed to control or prevent Newcastle Disease (ND). In some countries it is a notifiable disease requiring special control measures, enforced by law. Such enforcement requires clear definition of the disease (*Survashe* and *Desmukh*, 1998). Where the disease is endemic, control can be achieved by good hygiene combined with immunization (*Murphy et al*., 1999).

1.11.1 Vaccination against Newcastle disease virus

For most infectious diseases vaccination is the method of choice for prevention. Vaccination for protecting chickens from Newcastle disease is routinely practiced through out the world (*Rahman et al*., 2002). Both live-virus vaccines containing naturally occurring lentogenic virus strains and inactivated virus (injectable oil emulsions) being commonly used (*Murphy et al*., 1999 and *Seal et al*., 2000). These vaccines are effective and safe, even in chicks, and may be administered via drinking water or by aerosol, eye or nostril droplets, or beak dipping. Laying hens are revaccinated every four months (*Murphy et al*., 1999).

1.11.1.1 Live –virus vaccines

These vaccines are prepared from live attenuated viruses and capable to infect cells. Strains of virus of low or moderate virulence are used. They mimic natural infection and induce all three immune responses (*Grimes*, 2002). The superior protection against Newcastle disease afforded by aerosol

administration of live-virus vaccines is well documented (*McMullin*, 1985and *Tabidi et al*., 2004).

1.11.1.1.1 Mesogenic vaccine strains

1.11.1.1.1.1 H. Strain

Ayar and Dobson in 1946 (cited by *Survashe* and *Desmukh*, 1998) passaged the field isolate H through chick embryos and attenuated it to use as vaccine strain.

1.11.1.1.1.2 Roakin strain

It is naturally occurring mesogenic field isolate and is also used as mesogenic vaccine (*Survashe* and *Desmukh*, 1998).

1.11.1.1.1.3 Mukteswar strain

Ayer and Dobson in 1940 (cited by *Survashe* and *Desmukh*, 1998) carried out the attenuation of Ranikhet strain in India and further work was continued in India by Hadow and Idnani. At 115 to 126 embryos passage the vaccine is used for flock vaccination. Later on after few more passages the strain was designated as 'Mukteswar strain' and now used in India and other Asian countries effectively to control ND (*Survashe* and *Desmukh*, 1998). Mukteswar strain is an invasive strain, it used as a booster vaccine although it can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. The vaccine is usually administered by injection (*Grimes*, 2002).

1.11.1.1.1.4 Komorov strain

This strain was produced by Dr Heifa Komorov in 1946 by serial intracerebral passages of field isolate in ducklings (*Survashe* and *Desmukh*, 1998). This strain is less pathogenic than Mukteswar and used as booster vaccine. It is usually administered by injection (*Grimes*, 2002).

1.11.1.1.2 Lentogenic vaccine strains

1.11.1.1.2.1 F strain

The F strain was first reported by Asplin in 1952 (cited by *Survashe* and *Desmukh*, 1998) in England. It is closely related to B1 strain. This vaccine is usually used in young chickens but suitable for use as a vaccine in chickens of all ages (*Grimes*, 2002).

1.11.1.1.2.2 Hitchner (HB1) strain

The Hitchner (HB1) or B1 (Bulksberg strain) was first described by Hitchner in 1948 and used as vaccine (*Survashe* and *Desmukh*, 1998).

1.11.1.1.2.3 LaSota strain

This strain was originally isolated by Beaudette in 1946 and used as vaccine strain (*Survashe* and *Desmukh*, 1998). This strain often causes post vaccination respiratory signs and used as a booster vaccine in flocks vaccinated with F or B1 (*Grimes*, 2002).

1.11.1.1.3 Avirulent vaccine strains

1.11.1.1.3.1 V4 strain

The V4-NDV is an avirulent strain of NDV used as vaccine in chickens of all ages. V4-HR is a heat resistant V4, it is thermostable and used in chickens of all ages (*Grimes*, 2002).

1.11.1.1.3.2 I-2 strain

I-2 NDV strain is thermostable and also used in chickens of all ages. Thermostable Newcastle disease vaccines exhibit a relative resistance to inactivation when exposure to elevated temperature (*Grimes*, 2002).

The mildly virulent B1 and La Sota strains of NDV are currently the most widely used efficacious live- virus vaccines for prevention of Newcastle disease and are marketed worldwide (*Seal et al*., 2000; *Shuaib et al*., 2006 and *Hofsade et al*., 1978). These live-virus vaccines induce high levels of IgA,

IgY and IgM antibodies in sera of newly hatched chicks. They also induce local antibody response such as IgA production in the Harderian gland along with lacrymal IgM following intraocular inoculation with NDV (*Seal et al*., 2000).

Vaccination programmes are adopted using live lentogenic and mesogenic ND vaccine strains. ND LaSota, ND B1 and ND F strains are commonly used for vaccination of young chicks at an early age. In general, LaSota vaccine gives better protection than B1 and also has a greater tendency to spread from bird to bird. Mesogenic vaccines like ND Mukteswar or Komorov strains are used later after eight weeks of age as a booster vaccination (*Survashe* and *Desmukh*, 1998). Several disadvantages exist, the most important that the vaccine may cause disease. Therefore, it is important to use extremely mild virus for primary vaccination. Maternally derived immunity may prevent successful primary vaccination with live virus (*Alexander*, 2003).

1.11.1.2 Killed (inactivated) vaccines

Since 1930 inactivated ND vaccines have been used for vaccinations (*Survashe* and *Desmukh*, 1998). The ability of the virus to infect cells has been destroyed by treatment with a chemical, radiation or heat. These vaccines invoke only a circulating antibody response (*Grimes*, 2002). Adjuvant vaccines enhance the immune response. Now a number of different oil emulsion vaccines are available as individual ND killed or combination with IB, IBD and are widely in use. Live priming and killed booster vaccination strategy is used to maintain high levels of antibody production to prevent infection and drop in egg production. In breeder flocks in addition to maintaining high levels of ND antibodies in the flock, it will also transfer

good level of maternal antibodies to their progeny (*Survashe* and *Desmukh*, 1998).

Inactivated oil- emulsion vaccines are not affected by maternal immunity as live vaccines and can be used in day- old chicks (*Alexander*, 2003).

1.11.1.3 Recombinant or subunit new generation vaccines

Several recombinant vaccines have been developed that provide protection against Newcastle disease (*Seal et al*., 2000). The present ND vaccines produced by conventional methods are still time-honored and also economical. However, using molecular biology technology the understanding of pathogenicity and the antigenicity of NDV has enabled cloning of the required gene in order to develop new generation vaccines for effective control of ND. Further research in developing subunit vaccines or recombinant vector ND vaccine is ongoing with encouraging results. Recently, a USA-based company has produced USDA approved fowl pox virus vector vaccine for the immunization of chickens against ND and fowl pox. It protects the birds without post vaccinal respiratory reactions (*Survashe* and *Desmukh*, 1998).

1.11.1.4 An '*in ovo*' vaccination

In ovo vaccination is an emerging trend in the poultry industry because of its advantages like negligible manpower involvement, induction of neonatal resistance and better protection. *In ovo* vaccination has been proved to be effective against Marek's disease (MD), and infectious bursal disease (IBD) of poultry (*Manna et al*., 2007). Research in this line for '*in ovo*'vaccination with ND vaccine is in progress and the chemically treated ND-B1 EMS vaccine strain has been developed. It is immunogenic and non-pathogenic to 18 day-old embryos. Chicks hatched from '*in ovo*' vaccinated eggs are

resistant to ND challenge up to four weeks of age. It is showing good potential and the work is still in progress (*Survashe* and *Desmukh*, 1998).

1.11.2 Vaccination Programs

Timing of vaccination of broiler chickens can be especially difficult due to the presence of maternal antibodies and the short life of the broilers. On the other hand vaccination of laying hens always requires more than one dose of vaccine to maintain immunity throughout their lives (*Alexander*, 2003). Laying hens are revaccinated every four months (*Murphy et al*., 1999).

Protection against NDV disease can be expected about a week after vaccination. Vaccinated birds excrete the vaccine virus for up to 15 days after vaccination. Furthermore, infected birds can shed wild-type virus for up to 40 days even after being vaccinated and may thus represent an important virus reservoir (*Murphy et al*., 1999).

In a study designed to compare different routes of Newcastle disease vaccination with B1 strain in day-old chicks, Eidsen and Kleven (1976) found that aerosol route provide the best protection, followed by the ocular route.

Despite the availability of a good number of conventional vaccines using strains like B1, F, Clone 30, La Sota, Mukteswar or other lentogenic/mesogenic strains of NDV, vaccination failure is common due to non-maintenance of cold chain, poor selection of vaccine strain, insufficient dose, presence of maternal antibody, and faulty vaccination- schedule (*Manna et al*., 2007). Failure of young chickens to develop expected levels of immunity after vaccination for Newcastle disease (ND), infectious bursal disease, and other diseases is often due to immune interference from passively acquired maternal antibody, which is transferred from hens to progeny via the egg yolk (*Stone et al*., 1992).

1.12 Isolation and purification methods of egg antibodies

There have been many studies regarding the isolation and purification of egg antibodies, especially considering the easy access to this source of antibodies and the high levels of specific antibodies present in the egg. Various chemicals have been used for the isolation of egg yolk antibodies (*Hamal et al*., 2006). Several methods can be used, even for large-scale purification, of functionally active chicken antibodies from egg yolk (*Carlander*, 2002). Chicken IgY isolated from egg yolk has a molecular weight (180 KDa) (*Raj et al*., 2004 and *Bizhanov et al*., 2004), which is higher than that of mammalian IgG (*BIžanov* and *Jonauskienė*, 2003).

There are several methods of purification of IgY described. The choice of method is a matter of yield and purity desired, final use of the IgY as well as material cost and labor skills (*Carlander*, 2002). Several methods were described in the 1950ies for purifying IgY based on the strategy of separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using extraction with organic solvents with rather low yields of antibody (*Bizhanov et al*., 2004). However, purification methods based on organic solvents like chloroform remain in use. Other methods are based on affinity chromatography or on dilution of the yolk followed by a freezing-thawing process after which the process consists of ion exchange chromatography and/or salt precipitations often combining a number of salts like e.g. polyethylene glycol (PEG) (*Bizhanov et al*., 2004 and *Hamal et al*., 2006), dextran sulfate, dextran blue, sodium sulfate, ammonium sulfate caprylic acid and sodium citrate. More recently methods combining chloroform removal of lipids with ammonium sulfate precipitation techniques have been shown to result in a good yield of antibodies of high purity (*Bizhanov et al*., 2004). Bade and Stegemann (1984) used isopropanol and acetone.

Four separation and purification methods in terms of yield, purity, ease of use, potential scaling up and immuno-activity of IgY were compared by Akita and Nakai in1993. The water dilution method (WD) was compared with three other methods, namely, polyethylene glycol (PEG), dextran sulphate (DS) and xanthan gum (Xan) techniques. The WD method gave the highest yield, followed by DS, Xan and PEG methods, in that order. 9.8 mg IgY/ml egg yolk was routinely obtained using the WD method, compared to 4.9 mg IgY/ml egg yolk with the popular PEG method with a purity of 94% and 89%, respectively. All these methods had no adverse effect on the immuno-activities of IgY. WD was also found superior in terms of ease of use and large scale production of IgY. WD method therefore provides a simple, rapid and efficient means of purifying IgY with high activity (*Akita* and *Nakai*,1993).

Isolation of IgY from the yolks of eggs by a chloroform polyethylene glycol procedure was done by Polson in 1990. He compared this procedure with the polyethylene glycol procedure which is currently being used. Polson found that the chloroform - polyethylene glycol method yielded 2.57 times more IgY than the conventional polyethylene glycol method (*Polson*, 1990).

It has been demonstrated that the IgY preparation with DS is very effective, quick and simple to perform. It is well-suited for use in combination with other methods, e.g. ammonium sulfate precipitation (*Szabo et al*., 1998).

Isolation of Immunoglobulin from Egg Yolk was done by using Anionic Polysaccharides and the IgY recovery was determined to be 33-74% by means of single radial immunodiffusion method when IgY was isolated under the optimal conditions (*Chang et al*., 2000). Purification of IgY from chicken egg yolk by preparative electrophoresis was done by Gee *et al* (2003). The IgY yield was greater than 80% by immunoassay.

Recently, the commercially available IgY purification kits provide a quick, simple and efficient method to extract IgY from egg yolk. They usually contain delipidation and precipitation reagents and require multiple step methods.

Chicken immunoglobulin Y(IgY) does not bind bacterial Fc receptors such as staphylococcal protein A or streptococci protein G or mammalian Fc receptors, as do most mammalian Immunoglobulins (*Raj et al*., 2004). Thus, classical affinity chromatography methods such as Protein A and Protein G cannot be used to purify IgY from egg yolks. In most of the work, enzyme linked immuosorbent assays (ELISA) were used to study the IgY activity. The ELISA principle is that an antigen is coated to a surface. The desired antibody in a sample is then allowed to attach itself to the antigen. The bound sample antibody is then detected by another, labeled antibody. The amount of the sample antibody can then be correlated to the labeled antibody in the assay (*Carlander*, 2002).

1.13 IgY as an alternative to mammalian antibodies

The immunoglobulin of egg yolk differs from mammalian IgG in the molecular size (larger), isoelectric point (more acidic), and has no binding ability with mammalian complement and protein A (*Hatta et al*., 1990). IgY can be used as an alternative to mammalian antibodies normally used in research, and its use in immunotherapy has recently been proposed. Compared to mammalian antibodies, IgY possesses several biochemical advantages and its simple purification from egg yolk prevents a stressful moment in animal handling, as no bleeding is necessary (*Carlander*, 2002). Due to present-day constraints regarding animal experimentation, chicken yolk IgY offers an easy and acceptable alternative for production of antiserum. Moreover, the active transport of IgY from serum to the egg occurs in a higher concentration than

in serum. Thus, more antibodies can be produced per month than in rabbits (*Raj et al*., 2004).

IgY is also known as γ-livetin and exists in egg yolk together with other two water- soluble proteins, α- livetin (chicken serum albumin) and β- livetin (α2 glycoprotein) and various lipoproteins (LDL and HDL) which are the major components of the egg yolk (*Hatta et al*., 1990). The major problem in the isolation of chicken antibodies (IgY) is the removal of lipids, which are present at high concentration in egg yolk (*Stålberg* and *Larsson*, 2001 and *Jensensius et al*., 1981). Egg yolk contains approximately 50% water. The dry weight of egg yolk is made up of 2/3 of lipids, and 1/3 of proteins (*Stålberg* and *Larsson*, 2001).

Egg can be stored for up to one year at 4°C prior to IgY purification (*Haak-Frendscho*, 1994). Typically, each egg will contain about 90-100mg of total IgY (*Carlander*, 2002 and *Haak-Frendscho*, 1994), and the specific antibody generally comprises 1-10% of that total, or about 1-10mg of specific IgY per egg (*Haak-Frendscho*, 1994).

The use of chicken egg yolk as a source for antibody production represents a reduction in animal use as chickens produce larger amounts of antibodies than laboratory rodents. It also makes it possible to eliminate the collection of blood, which is painful for the animal. The European Centre for the Validation of Alternative Methods (ECVAM) recommends that yolk antibodies should be used instead of mammalian antibodies for animal welfare reasons (*Carlander*, 2002). In 1999, the IgY-technology was approved as an alternative method for supporting animal welfare by the Veterinary Office of the Swiss Government, Office Vétérinaire Fédéral (*Schade et al*., 2005).

Chicken eggs have long been recognized as a potential source of pharmaceutical product and represent a low cost, high-yield bioreactor system.

Egg white and egg yolk are sterile; their proteins can be fractionated with different technologies and combined with the egg industry's capacity to produce thousands of eggs per day (*Narat*, 2003).

As a laying hen produces approximately 20 eggs per month, over two gram IgY per month can be isolated. The IgY concentration in chicken serum is approximately five-seven mg/ml, therefore two gram of egg yolk IgY corresponds approximately to the IgY content of 300 ml of serum or 600 ml of blood. Only larger mammals can produce equal amounts of serum antibodies and compared to rabbits, the chicken antibodies are ten times less expensive (*Carlander*, 2002).

Highly conserved mammalian proteins sometimes fail to illicit a humoral immune response in animals, such as rabbits, that are traditionally used for generating polyclonal antibodies. Since chicken IgY does not cross-react with mammalian IgG and does not bind bacterial or mammalian Fc receptors, non-specific binding is reduced, and the need for cross-species immunoabsorptions is also eliminated (*Haak-Frendscho*, 1994).

1.14 Application of IgY antibodies

There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons and chicken egg yolk antibodies (IgY) have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes (*Bizhanov et al*., 2004 and *BIžanov* and *Jonauskienė*,2003).

New application in human and veterinary medicine including strategies for the treatment of *Helicobacter pylori* infection or fatal intestinal diseases in children, particularly in poor countries, for reducing the use of antibiotics, and, in Asia and South America, for producing Abs against snake, spider and scorpion venoms (*Schade et al*.,2005).

Makvandi and Fiuzi found that hepatitis B virus (HBV) vaccine was immunogenic in hens and ELISA using the purified anti-HBsAg from the egg yolks was able to detect the HBsAg in the sera of patients infected with hepatitis B virus (*Makvandi* and *Fiuzi*, 2002).

Passive immunization by oral administration of specific antibodies has been an attractive approach against gastrointestinal (GI) pathogens in both humans and animals. Oral administration of IgY has proved successful for treatment of a variety of GI infections, such as bovine and human rotaviruses, bovine coronavirus, *Yersinia ruckeri*, enterotoxigenic *Escherichia coli, Salmonella* spp., *Edwardsiella tarda, Staphylococcu*s, and *Pseudomonas* (*Yoshinori* and *Kovacs-Nolan*, 2002).

A study done in 2006 demonstrated that hyperimmunized yolk can be raised to purify antibodies which can then be used to control infectious bursal disease (IBD) infected commercial layers, commercial broilers, broiler breeders and indigenous birds (*Malik et al*.,2006) .

CHAPTER TWO
MATERIALS AND METHODS

2.1 Collection of samples

Laying bovan chicken aging 5-8 months were used to collect eggs. The flock was vaccinated with Komorov strain of NDV. The last dose of the vaccine was at four month old. Ninety six egg samples were collected and placed at 4°C till examined.

2.2 Extraction of IgY from Egg Yolk by dextran sulphate method

Extraction was carried out by the protocol outlined by Jensenius *et al* (1981).

2.2.1 Reagents

Tris buffered saline containing 0.1% sodium azide (TBS)

10% (w/v) dextran sulphate in TBS

1M calcium chloride in TBS

Sodium sulphate, anhydrous.

36 %(w/v) sodium sulphate.

2.2.2 Materials required

Clean Petri dish, forceps, 50ml graduated centrifuge tubes, glassware such as flasks and beakers, pipetting device with disposable pipette tips and dialysis tube with molecular weight cut off 12-14000 Dalton (Medicell International Ltd,UK).

2.2.3 Isolation procedure

To extract IgY from the egg yolk, the egg yolk was taken out of the eggshell and placed in a clean Petri dish. The egg yolk membrane was broken with the help of forceps. The yolk was allowed to run into a 50ml graduated centrifuge tube, and its volume was noted after it settled down. Tris buffered

saline (TBS) containing 0.1% sodium azide was added to the yolk to bring the volume to 50ml and mixed. Diluted yolk suspension was centrifuged at 2000 g for 20 min at 20°C. Pellet was discarded and supernatant was saved. Supernatant was mixed with 3 ml of 10% dextran sulphate solution and 7.5 ml of 1M calcium chloride solution; incubated for 30 min and then centrifuged as above. Clear supernatant was saved. The precipitate was resuspended in 50 ml of TBS and recentrifuged. The two supernatants were pooled and the final pellet was discarded. If the first supernatant was cloudy, more dextran sulphate and calcium chloride were added and incubation and centrifugation were repeated. TBS was added to the pooled supernatants to the final volume of 100 ml and 20 g of anhydrous sodium sulphate was slowly added, stirred to dissolve and incubated at room temperature for one hour then centrifuged at 2000 g for 20 minutes at 25°C. The supernatant was discarded. The protein precipitate was redissolved in TBS to the final volume of 10 ml. Sodium sulphate solution was added to make a final concentration of 9% (w/v),(=2.5 ml of the 36% Sodium sulphate solution) ,mixed and incubated for one hour at room temperature. After incubation period, the mixture was centrifuged at 2000g for 20 minutes at 25°C and the pellet was discarded. Sodium sulphate solution was added to the saved supernatant to make a final concentration of 14 %(w/v), (=3.9 of the 36% Sodium sulphate solution), mixed and incubated for one hour at room temperature. After incubation period, the mixture was centrifuged at 2000g for 20 minutes at 25°C. The supernatant was discarded and the Pellet (immunoglobulin precipitate) was redissolved in 5 ml TBS and dialyzed against TBS with dialysis volume approximately 50 times larger than the sample volume for 24 hours. After dialysis, the sample was poured in sterile 5ml tube and stored at 4°C till use. Specificity of IgY to NDV was determined by standardized indirect ELISA (In- house ELISA).

2.3 Determination of the specificity of the extracted IgY to NDV by standardized indirect Enzyme -Linked Immunosorbent Assay (ELISA) (In-house ELISA)

2.3.1 Materials required:

96-well Flat-bottomed ELISA plates (Nunc-Immunoplate, Denmark)

NDV vaccine (Komorov strain) as coating antigen (Central Veterinary Research Laboratories center, Khartoum, Sudan)

Samples under test (extracted IgY)

0.05M Carbonate buffer, pH 9.6

0.15M Phosphate buffered saline (PBS), pH 7.2

0.05M Phosphate-citrate buffer, pH 5.0

Tween 80

Bovine serum albumin (BSA) (Sigma, USA)

Horseradish peroxidase-conjugated rabbit anti-chicken IgG (Nordic immunological laboratories, the Netherlands)

O-phenylenediamine dihydrochloride (OPD) (Sigma immuno chemicals, USA).

30% hydrogen peroxide solution

1N hydrochloric acid (HCl)

Positive control serum (prepared in the lab)

Negative control serum (PBS buffer was used)

Single and multichannel pipettes and tips

V-bottom troughs

ELISA reader

2.3.2 Preparation of ELISA solutions

2.3.2.1 Carbonate Coating buffer

Coating buffer used for antigen dilution was 0.05M Carbonate buffer, pH 9.6. It was prepared by dissolving 1.59g sodium carbonate (Na_2CO_3) and 2.93g sodium hydrogen carbonate ($NaHCO_3$) in one liter of distilled water then pH was adjusted.

2.3.2.2 Washing buffer

0.15M Phosphate buffered saline (PBS), pH 7.2 was prepared by dissolving 8.00g sodium chloride (NaCl), 0.20g potassium chloride (KCl), 1.15g di-sodium hydrogen phosphate (Na_2HPO_4) and 0.20g potassium di hydrogen phosphate (KH_2PO_4) in one liter of distilled water and the pH was adjusted.

2.3.2.3 Substrate buffer

0.05M Phosphate-citrate buffer, pH 5.0 was prepared by adding 25.7ml of 0.2M di-sodium hydrogen phosphate (Na_2HPO_4) to 24.3ml of 0.1M citric acid and completed to 100ml by adding 50ml deionized water. The pH was adjusted. This buffer was used for substrate preparation.

2.3.2.4 Blocking solution

Blocking solution was 2% Bovine serum albumin (BSA) in PBS (website 1).

2.3.2.5 The diluent

The diluent for primary and secondary Abs was PBS containing 0.5% BSA and 0.05% Tween 80 (PBS- Tween) (website 2).

2.3.2.6 The conjugate

The conjugate Horseradish peroxidase-conjugated rabbit anti-chicken IgG was purchased from Nordic labs (Tilburg, The Netherlands). It was lyophilized and was reconstituted by adding 1ml sterile distilled water and that gave 1ml of IgG protein with concentration of 4.6mg/ml. The reconstituted conjugate was divided into small aliquots each one contains 0.1ml (100 µL)

kept frozen at -20°C. Prior to use, an aliquot was thawed slowly at ambient temperature and used to prepare working dilution by adding sterile Phosphate buffered saline (PBS), pH 7.2.

2.3.2.7 The substrate

The substrate O-phenylenediamine dihydrochloride (OPD) was used. The 30mg substrate tablet was dissolved in 75ml 0.05M Phosphate-citrate buffer, PH 5.0 to give a concentration of 0.4mg/ ml as recommended by the manufacturer. 40 µL of fresh 30% hydrogen peroxide per 100ml of substrate buffer solution was added immediately prior to use.

2.3.2.8 Stopping solution

The 1N HCl was used for stopping the reaction. 85.85 ml of 36%HCl was completed to one liter by adding distilled water to give 1N HCl.

2.3.3 Preparation of hyper-immune serum against NDV as positive control

2.3.3.1 The vaccine:

The freeze-dried live chick-embryo adapted vaccine containing Komorov strain (K) of NDV was used. The vaccine was kindly supplied by the Viral Vaccine Department; Central Veterinary Research Laboratories center (Khartoum Sudan). It was kept at 4°C till use.

2.3.3.2 Immunization of chickens

Four laying hens from ND-vaccinated flocks (with Komorov strain of NDV) were boosted intramuscularly in the breast with 0.2ml of 1:1000 and 1:100 respectively with two weeks interval .Two weeks later, 3ml blood was collected from each hen from the wing vein using 5ml sterile syringes. Blood was then left in syringes undisturbed at room temperature for three hours. Any expressed serum was collected and stored in Eppen Dorf tubes at 4°C to be tested by haemagglutination- inhibition test to for NDV antibody titeration.

2.3.3.3 Haemagglutination- inhibition test

2.3.3.3.1 Viral antigen

The vaccine containing Komorov strain (K) of NDV was titrated to be used as the antigen in the HI test. The standard amount of Newcastle disease virus used in the haemagglutination inhibition (HI) test was 4HA units.

2.3.3.3.2 Determination of 4HA units of Newcastle disease virus antigen by haemagglutination test:

2.3.3.3.2.1 Materials required

96-well microtitre plates, U-shaped

1% chicken red blood cell (RBC) solution

Phosphate-buffered saline (PBS), pH 7.2

2.3.3.3.2.2 Preparation of chicken red blood cells (CRBCs)

Five ml blood was collected from an adult bird in a test tube containing 0.5 ml of a 4 % sodium citrate as anticoagulant. The blood was centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. Packed RBCs were resuspended in 10 ml of phosphate buffered saline (PBS).The process of RBCs washing in PBS was repeated until a clear supernatant was obtained. The packed RBCs was resuspended in PBS to give 1 % RBCs suspension.

2.3.3.3.2.3 Haemagglutination (HA) titration procedure

Fifty µl of PBS (pH 7.2) was added to all wells in the first four rows except column 1. 100 µl of the antigen was added to column1 wells. Using multichannel pipette, serial 2-fold dilution was made by transferring 50 µl from the first wells of lettered rows to successive wells. The final 50 µl was discarded. The D row was prepared as RBC control wells by adding 100 µl of PBS (No antigen was added). 50 µl of 1% RBC suspension was added to all wells. Then plate was shaken for about five seconds on a microshaker, then incubated at room temperature and read after 30 minutes or as soon as the

RBCs in the control wells had settled. The end-point was taken as the dilution in the last wells that showed complete (100 percent end point) haemagglutination. The HA titer was the reciprocal of that value. The antigen dilution (4HA units of Newcastle disease virus antigen) was calculated by dividing the HA titer by 4. The diluted antigen containing 4HA units was stored at 4°C and used within the same day (*Figure* 5).

2.3.3.3.2.4 Haemagglutination- inhibition test procedure

2.3.3.3.2.4.1 Materials required

U-bottom microwell plates and covers

Phosphate buffered saline (PBS), PH 7.2

1% chicken red blood cell (RBC) solution

V-bottom reagent trough

50 µL single and multichannel pipettes and tips

Recording sheet.

Newcastle disease virus antigen diluted to 4 HA units

2.3.3.3.2.4.2 Test procedure

Recording sheet was filled in to record how samples will be dispensed into microwell plate. Rows from A to F was used. 50 µL of PBS was dispensed into all wells. 50µL of each serum sample was dispensed into each well of the first column (ABCDF) then a multichannel pipette was used to make two-fold serial dilutions along the rows, the last 50µL was discarded. The wells of row G was prepared as negative control wells by adding 100 µL of PBS (no Abs added). 50 µL of the 4HA dilution of antigen was added to all wells including the control. Plate was shaken to mix the reagents, covered with a lid and allowed to stand for 30 minutes at room temperature. 50 µL of 1% washed red blood cells was distributed into all wells including the control, covered and incubated at room temperature for 45 minutes and the

results were read. The titer of haemagglutination-inhibiting antibodies was the highest serum dilution that inhibited the agglutination of 75% −100% of red blood cells and expressed as the reciprocal of that dilution (*Figure* 6).

2.3.3.3.4 Production of positive control serum

Three of chickens immunized by ND vaccine gave antibody titer (7 $\log_2$) in HI test and were selected to be used as positive controls. 10ml blood was collected from each selected hen from the wing vein using 10ml sterile syringes and immediately poured into a sterile centrifuge tubes. Blood was allowed to clot at room temperature (about one hour) and left overnight at 4°C. Clot was detached from walls of the container and all clot free serum was poured into a centrifuge tube, clot was centrifuged for 30min at 2500g at 4°C and any expressed serum was removed and pooled with the first liquid. The pooled sera were centrifuged for 20 min at 1500g at 4°C. Sera obtained were pooled together (about 15ml), divided into small aliquots and stored at -20°C tell use.

2.3.4 Selection of ELISA plates

High protein-binding capacity polystyrene 96-well Flat-bottomed ELISA plates from Nunc (Denmark) were used since low-protein binding capacity plates yield low signal or inconsistent data.

2.3.5 Standardization of ELISA reagents

Optimal dilutions of the antigen and the conjugate were estimated by checkerboard titration as follow:

2.3.5.1 Antigen preparation

The freeze - dried, live, chick- embryo adapted vaccine containing Komorov strain (K) of NDV (Centre of Veterinary Research Laboratories, Sudan) was used as coating antigen. The antigen was lyophilized. It was reconstituted in one ml carbonate buffer and then diluted as specify.

Fig. 5: Haemagglutination test plate.

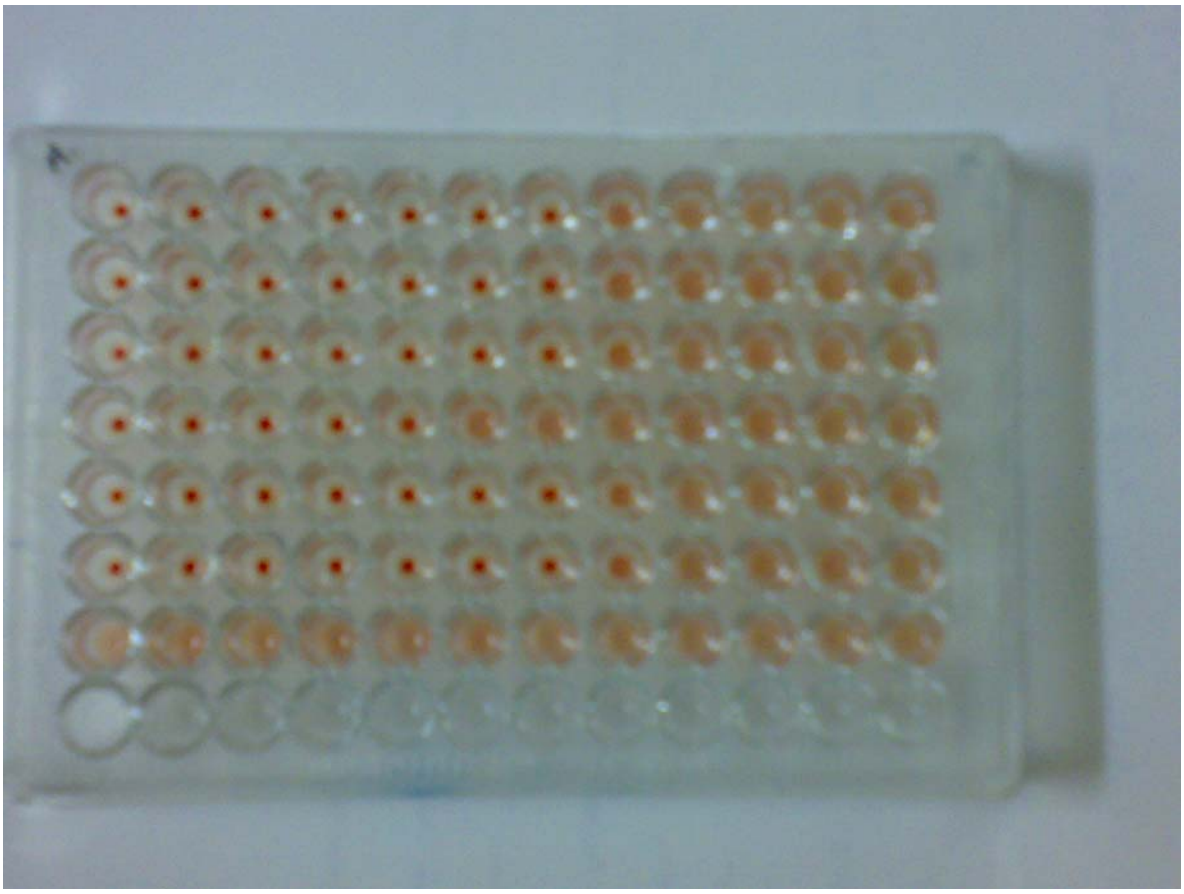


Fig. 6: Hemagglutination- inhibition test plate.

Rows (ABCDEF) contain PBS, serum, antigen and 1% chicken RBCs. Row G contains PBS, 4HA units antigen and 1% chicken RBCs as control.

2.3.5.2 Titration of the antigen

Two flat-bottomed polystyrene microtiter plates were coated with 100µl/well of increasing dilutions of antigen, from1:10 to1:80 in the first plate and from 1:90 to1:160 in the second plate, each row contained one dilution. The carbonate buffer was used for antigen dilution. The plates were incubated for one hour at 37°C and then over night at 4° C, and washed three times with PBS.

2.3.5.3 Titration of the serum

One ml of hyperimmune serum was diluted in nine ml PBS-Tween to make 10ml of dilution 1:10. In each one of the two precoated plates, 100µl/well of diluted serum was distributed in columns 1- 3, 5- 7 and 9- 11. Negative control wells were prepared in columns 4, 8 and12 by adding 100µl/well PBS without serum. Plates were incubated for one hour at 37° and then washed three times with PBS.

2.3.5.4 Titration of the conjugate

The secondary antibody, a peroxidase-conjugated rabbit anti-chicken Immunoglobulin was prepared in concentrations of 1:1000, 1:5000 and 1:10000 (diluted in PBS-Tween) and distributed in 100 µl volume per well in each plate (1:1000 from column1 to 4, 1:5000 from column 5 to column 8 and1:10000 from column 9 to column 12). Plates were incubated for one hour at 37° C. The plates were then washed three times with PBS (*Figure* 7).

Finally, 100µl/well of peroxidase substrate (OPD) was added to each well and incubated for 20 minutes at room temperature. The optical density (OD) was read with the ELISA reader at 450nm.

From the checkerboard experiments the optimal dilutions were found to be 1:50 for the antigen and 1:5000 for the conjugate (*Figure* 8).

	Serum dilutions →												
Antigen dilutions		1	2	3	4	5	6	7	8	9	10	11	12
↓	A												
	B												
	C												
	D												
	E												
	F												
	G												
	H												
	Conjugate dilutions →												

Fig. 7: Checkerboard titration plate format.

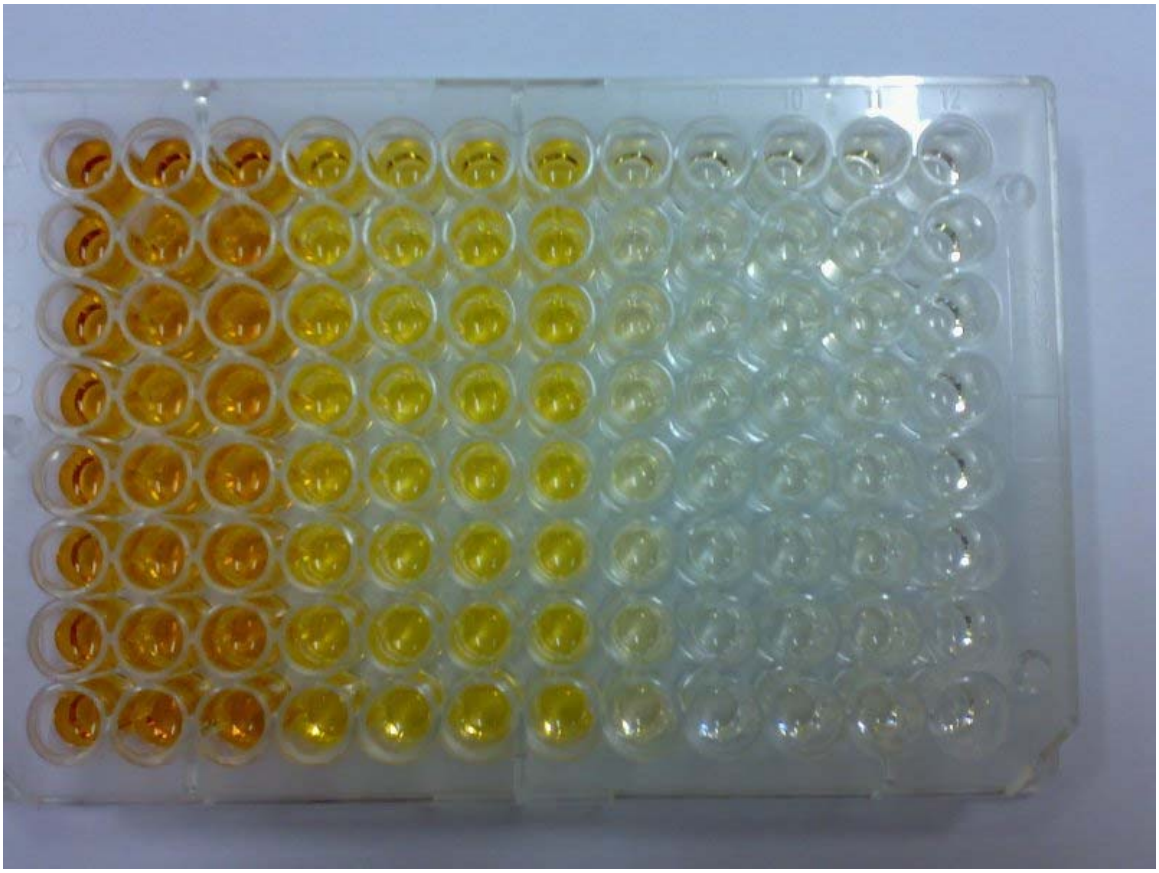


Fig. 8: Checkerboard titration plate.

2.3.6 Indirect ELISA for detection of presence of NDV antibodies in egg yolk (In-house ELISA)

Flat-bottomed ELISA plate was coated by dispensing 100 µL of antigen diluted to 1:50 in 0.1 M carbonate buffer pH 9.6 into all wells. The plate was incubated at 37°C for one hour and then over night at 4°C. The plate was then washed by filling all wells with 200 µL PBS using multichannel pipette. The plate was shaked gently and the content was poured out, the excess wash buffer was removed by banging the plate upside down on dry paper towels. Washig procedure was repeated three times. The plate was then blocked with 150 µL of 2 % bovine serum albumin in PBS for one hour at 37°C. The blocking was done to prevent the antibody to attach to any possible unbound sites then plate was washed three times with PBS as above. 100 µL of undiluted samples were added to wells in column 1. All test samples were diluted to 1:100 by adding 10 µL of sample to 990 µL of diluent and added to wells in column 2. Two- fold dilutions for each sample was made across the corresponding row to give 1:200, 1:400……etc using multichannel pipette starting at column 2 through to column 10. Tips used were changed after each transfer. The column 11 was used as a positive control. 1:100 dilution of the positive control serum was prepared (as done for samples above) and 100 µL of diluted positive control serum was added to each well in column 11. The column 12 was used as a negative controls by adding 100 µL/well PBS, no serum was added. The plate was incubated for one and a half hour at 37°C and was washed three times with PBS as previously described. Then 100 µL of the conjugate, (rabbit anti-chicken IgG-HRP) diluted to1:5000 in PBS-Tween was added to the plate and the plate was incubated for one hour at37°C. The plate was washed as previously described. The OPD, a horseradish peroxidase substrate was prepared and 30 µL of 30% hydrogen peroxide (H_2O_2) per

100ml of substrate buffer solution was added. 100 µL/well OPD was then added into plate and the plate was covered. The reaction was stopped after 15 minutes by addition of 100 µL of 1N HCl. The plate bottom wiped to dry. The intensity of color development was determined by measuring absorbance using a micro-ELISA reader (Rayto, RT- 6100, China) equipped with a 492 nm filter.

2.3.7 Detection of maternal specific NDV IgY using NDV ELISA test kit (comparative study)

2.3.7.1 Reagents included in the ELISA test kit

NDV coated plates

NDV positive control

Negative control

Goat anti- chicken horseradish peroxidase conjugate

Sample diluent buffer

TMB substrate

Stop solution

2.3.7.2 The test procedure

NDV antibody test kit (Flockchek-IDEXX, USA) was used to detect NDV antibody in chicken egg yolk. The test was performed as described by the manufacturer with some modifications. In brief, 100µl of undiluted negative and positive control were put into well AI, 2 and A3, 4 (of the coated plates) respectively. In the rest of the plate, 100µl of undiluted samples were added to each used well. The plate was incubated for 30 minutes at room temperature. Each well in the plate was then washed three to five times with approximately 350µl deionized water. 100µl of (goat) antichicken horseradish peroxidase conjugate was dispensed into each well and incubated for 30 minutes at room temperature. The plate was washed as mentioned above. 100µl of TMB substrate

solution were dispensed into each well then incubated for 15 minutes at room temperature. 100µl of stop solution were dispensed into each well to stop the reaction. The absorbance of the control serum and serum samples was recorded using the plate reader at 650nm. The software (Flockchek-IDEXX, USA) was used for calculation of sample to positive (S/P) values.

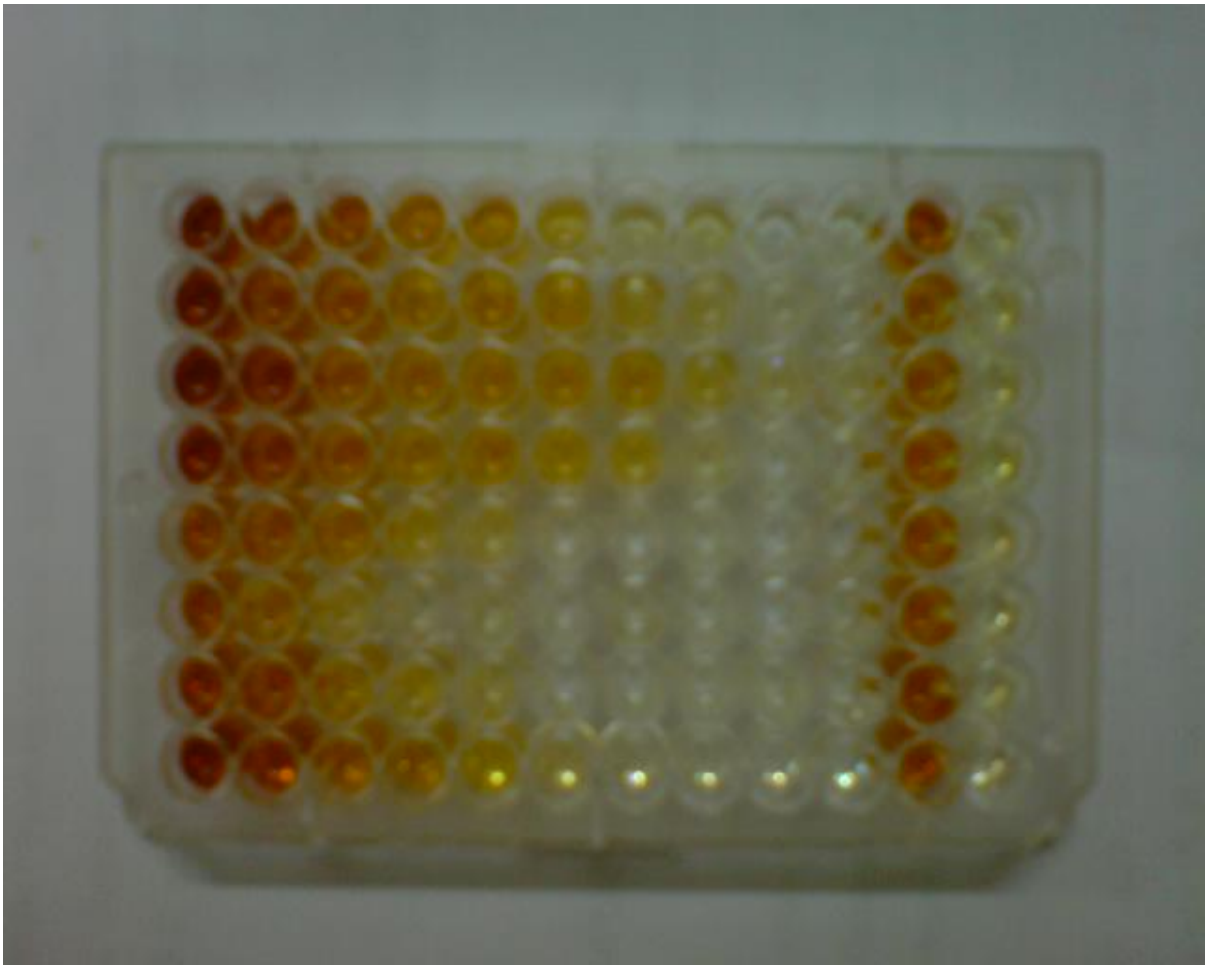


Fig. 9: Standardized indirect ELISA plate

CHPTER THREE

RESULTS

3.1 Detection of presence of NDV antibodies in the egg yolk by Standardized indirect ELISA

3.1.1 Interpretation of results

3.1.1.1 Determination of the cut-off value (the negative- positive threshold)

The cut-off value used for determination the presence or absence of antibodies to NDV in samples (positive or negative) was the positive- negative threshold which calculated as three standard deviations (SD) above the mean optical density of the negative control (m NC) as described by Snyder *et al* (1983):

The positive- negative threshold = m NC+ 3SD

All test samples values above the cut-off value were reported as positive.

3.1.1.2 The antibody titer

The antibody titer was expressed as the reciprocal of the lowest dilution giving an absorbance three times higher than the standard deviation of the negative control.

Results of each sample (OD, positive or negative and titers) are shown in Table (1) appendix 1.

3.2 Antibody titration by indirect ELISA kit

For the assay to be valid, the difference between the positive control mean and the negative control mean should be greater than 0.075.

The relative amount of antibodies in tested samples was determined by calculating the ratio of sample to positive control (S/P ratio).

$$S/P = \frac{\text{sample OD – negative control mean}}{\text{positive control mean - negative control mean}}$$

Samples with S/P ratio of less than or equal to 0.2 were considered negative and those with S/P ratio greater than 0.2 were considered positive. The endpoint titers were calculated using the following equation:

Log10titre = 1.09 (Log10 S/P) +3.36

Results of each sample (OD, positive or negative and titers) are shown in Table (2) appendix 2.

Log_2 of each sample titer was calculated using computer soft were then samples were grouped in titer groups as shown in table (3) and *figure* (10) for the In-house ELISA and in table (4) and *figure* (11) for the indirect ELISA kit.

Table (5) shows comparative results between the In-house ELISA and the indirect ELISA kit.

Table 3: Standardized indirect ELISA titer groups (In- house ELISA)

Titer group	Samples count	Titer (log 2)
1	12	8.643
2	7	9.643
3	15	10.643
4	16	11.643
5	23	12.643
6	17	13.643

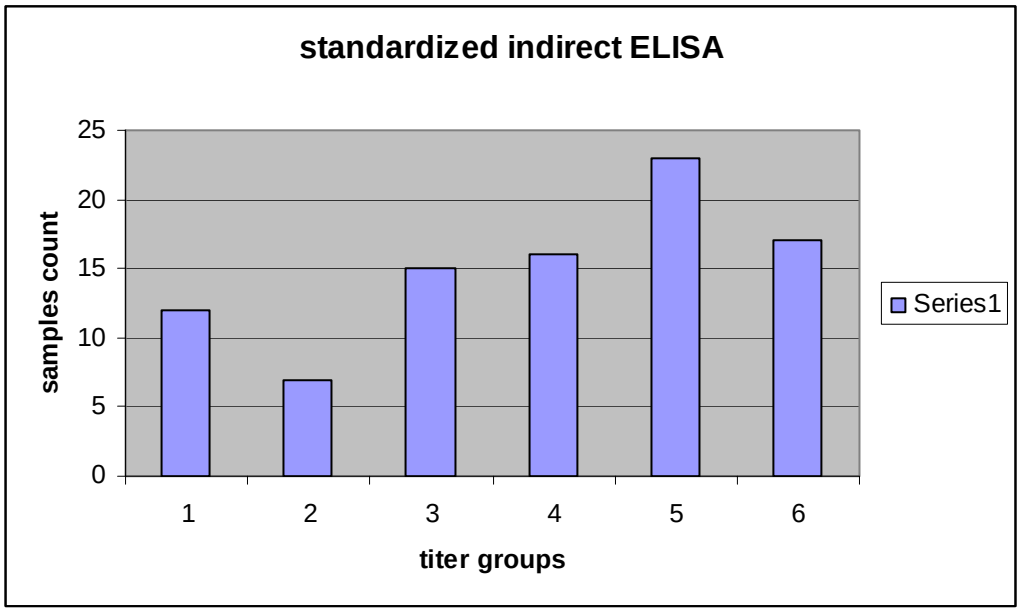


Fig. 10: Standardized indirect ELISA titer groups

Table 4: NDV ELISA test kit titer groups

Titer group	Samples count	Titer (log 2)
1	1	10.838
2	8	11.513 – 11.963
3	49	12.009 – 12.987
4	33	13.154 – 13.949
5	1	14.171

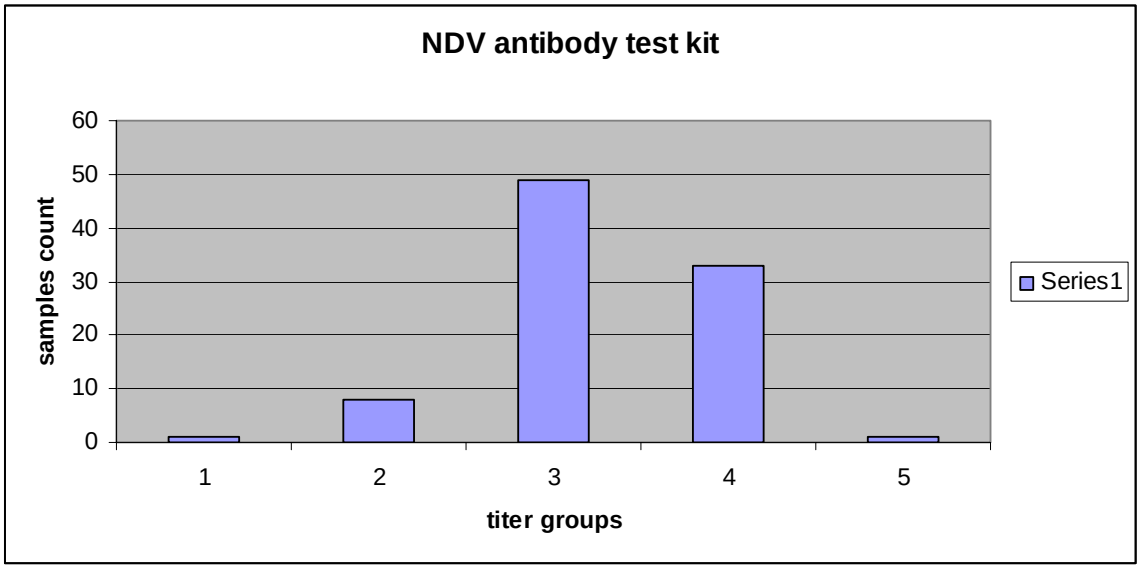


Fig. 11: NDV ELISA test kit titer groups

Table 5: Comparison between results obtained from the standardized indirect ELISA (In- house ELISA) and from the NDV ELISA test kit

	Samples number	Positive samples	Negative samples	Max Titer (log 2)	Min Titer (log 2)	Samples number with Titers (10 – 13)
Standardized ELISA	96	96 (100%)	0	13.643	8.643	71 (73.3%)
ELISA kit	92	92 (100%)	0	14.171	10.838	91 (98.8%)

CHAPTER FOUR

DISCUSSION

In this study the egg yolk antibodies were extracted by the dextran sulphate method. Szabo *et al* (1998) demonstrated that the IgY preparation with dextran sulphate is very effective, quick and simple to perform. This procedure does not give completely pure IgY but the preparation is free from other classes of immunoglobulin because of their absence from yolk as stated by Hamal *et al* (2006) who reported that IgY is present predominantly in the egg yolk, whereas IgA and IgM are present in the egg white and with Carlander (2002) who reported that IgA and IgM are not present in the yolk.

Chicken immunoglobulin Y (IgY) does not bind bacterial Fc receptors such as staphylococcal protein A or streptococci protein G or mammalian Fc receptors, as do most mammalian Immunoglobulins as reported by Raj *et al* (2004). Thus, classical affinity chromatography methods such as Protein A and Protein G cannot be used to purify IgY from egg yolks. Further purification can be carried out using the gel filtration on Sephadex G 200. Generally, the choice of method is a matter of yield and purity desired, final use of the IgY as well as material cost and labour skills.

In the present study presence of NDV antibodies in the egg yolk extracts (the positive results) indicates transfer of maternal antibodies from hen′s serum to the egg yolk and specificity of the antibodies to NDV.

Typically, each egg will contain about 90-100mg of total IgY and the specific antibody generally comprises 1-10% of that total, or about 1-10mg of specific IgY per egg as reported by Haak-Frendscho (1994) and Carlander (2002). Hamal *et al* (2006) reported that the IgY levels, total or antigen-specific, in the dams' plasma or eggs were found to be a direct indicator of

maternal antibody transfer to the chicks' circulation, with an expected percentage transfer of approximately 30%.

The size of the collected eggs was not the sam and Carlander (2002) reported that the amount of IgY transported is independent of egg size. The populations of IgY are transported according to their concentration in the maternal serum. There is no selection nor destruction of IgY during transport. Thus titers of the hen's serum antibodies to ND and the passive antibodies in chicks could be calculated indirectly by determining the titers of IgY in yolks.

Although maternal antibodies provide passive immunity in young chicks for a few weeks after birth and considered to be protective, they also interfere with the chick's active response to vaccination. Maternal antibody interferers with active immunization, presumably by sequestering vaccine antigen or restricting replication of vaccine virus, this was reported by Stone *et al* (1992). Rahman *et al* (2002) reported that maternal antibody neutralizes the introduced vaccine antigen rendering the vaccine ineffective and immune response was nil at high titer of maternal antibody and thus, taken into consideration during primary vaccination.

In this study high titers of NDV specific antibodies was measured in eggs of immunized hens collected during the first month after the last dose of vaccination (five months old). This booster dose in mothers developed high titers of NDV specific antibodies that peak at about three to four weeks after vaccination and these results substantiate the study reported by Alexander (2003). However the titers then slowly decrease as reported by Butcher and Miles (2003) and this explain the low titers in some egg yolk extracted from eggs of eight month old hens (three months after vaccination). This suggests the importance of revaccination of laying hens every four months as reported by Murphy *et al* (1999). Therefore, extraction of maternally derived NDV

antibodies will facilitate accurate monitoring of ND vaccination programmes. Furthermore antibody titer should not necessarily be high but should be in close range with the same herd.

As we demonstrated a considerable variation in maternal NDV specific antibody titer by both ELISA methods, we were able to place the ELISA results into NDV antibody ELISA titer groups. This lend us to suggest that determination of antibody titer in yolk or sera of laying hens or parent stock can facilitate batching of the breeding flock or laying hens into groups. This because egg or sera with low titer or no antibodies to the specific disease might constitute a threat of that disease as the degree of resistance induced by any vaccine is subject to the level of challenge present in poultry farms.

Intriguingly, in the present study the NDV antibody titers obtained in some of yolk extracts were much higher than the titer obtained from our prepared hyper-immune serum and this is in agreement with Schade *et al* (1996) who reported that the data available indicate that IgY is more highly concentrated in yolk than it is in serum and also in agreement with Raj *et al* (2004) who reported that the active transport of IgY from serum to the egg occurs in a higher concentration than in serum.

As a comparison, antibody titers obtained from commercial NDV antibody test kit were higher than those obtained from our standardized indirect house ELISA. This result indicates that the sensitivity of the NDV antibody test kit was higher than the standardized indirect (In- house) ELISA. In both tests, most samples have titers between 10 log 2 and 13 log 2. Both results were based on the optical density rather than absolute values (ng IgY/ml). Nevertheless, indirect ELISA remains an important tool to monitor the efficacy of vaccination where immunity is measured by antibody response as reported previously by Snyder *et al* (1983).

On conclusion, in this study maternally derived antibodies present in the egg yolk specific to NDV were present in high titers that confer protection during early weeks of life of hatching chicks. Extraction of maternally derived specific antibodies from egg yolk will facilitate accurate monitoring of ND vaccination programmes.

CONCLUSION

1- Dextran sulphate method for the extraction of egg yolk IgY antibodies was simple to perform and gave high recovery and purity of egg yolk IgY.

2- High titers of NDV specific IgY were detected in egg yolk of vaccinated hens, both by our In- house ELISA and the commercial NDV antibody ELISA kits.

3- Results obtained by In- house ELISA assay were comparable to the results obtained by the commercial NDV antibody ELISA kits.

4- The high titers of maternally derived NDV specific IgY could confer protection in newly hatched chicks.

5- Extraction of maternally derived NDV specific antibody from egg yolk of vaccinated hens will facilitate accurate monitoring of ND vaccination programmes.

6- Indirect ELISA assay can be used to monitor the efficacy of ND vaccines in poultry farms.

RECOMMENDATIONS

1- Dextran sulphate method for extraction of egg yolk IgY is simple procedure to isolate maternally derived IgY to evaluate maternal immunity in poultry farms.

2- Indirect In-house ELISA could be elaborated to be routinely used instead of expensive commercial ELISA kits to monitor the efficacy of vaccination where immunity is measured by antibody response.

3- Extraction of maternally derived specific antibodies will facilitate and accurate monitoring of vaccination programmes in poultry farms.

4- Determination of antibody titer in egg yolk or sera of hens and parent stock can facilitate separation these flocks into groups according to their immune status to reduce losses during epidemics.

5- Barring procedural errors in performing ELISA assay, scoring of samples with optical density reading skirting the cut off range are problematic compared to data expressed in absolute values derived from calibration curve. Absolute values are meaningful and acceptable and we recommend that the expression of ELISA assay in absolute values in future work.

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APPENDIX 1

Table 1: Maternal specific NDV antibody titer in individual egg measured by standardized indirect ELISA (In- house ELISA)

Sample number	Absorbance (OD)	result	titer	Log 2
1	0.987	pos	12800	13.643
2	0.796	pos	3200	11.643
3	1.109	pos	12800	13.643
4	0.928	pos	6400	12.643
5	1.004	pos	12800	13.643
6	1.392	pos	12800	13.643
7	0.967	pos	12800	13.643
8	0.865	pos	6400	12.643
9	0.642	pos	800	9.643
10	0.561	pos	400	8.643
11	0.774	pos	1600	10.643
12	0.672	pos	800	9.643
13	0.769	pos	3200	11.643
14	0.577	pos	400	8.643
15	0.901	pos	6400	12.643
16	0.695	pos	800	9.643
17	0.742	pos	1600	10.643
18	0.794	pos	3200	11.643
19	0.568	pos	400	8.643
20	0.701	pos	800	9.643

21	0.717	pos	1600	10.643
22	0.765	pos	1600	10.643
23	0.849	pos	3200	11.643
24	0.779	pos	3200	11.643
25	0.866	pos	6400	12.643
26	0.903	pos	6400	12.643
27	0.706	pos	1600	10.643
28	0.878	pos	6400	12.643
29	0.906	pos	6400	12.643
30	0.887	pos	6400	12.643
31	0.864	pos	6400	12.643
32	0.971	pos	12800	13.643
33	0.898	pos	6400	12.643
34	0.911	pos	6400	12.643
35	0.916	pos	6400	12.643
36	1.002	pos	6400	12.643
37	0.612	pos	400	8.643
38	0.964	pos	12800	13.643
39	0.853	pos	6400	12.643
40	0.866	pos	6400	12.643
41	0.782	pos	3200	11.643
42	0.793	pos	3200	11.643
43	0.844	pos	3200	11.643
44	0.762	pos	1600	10.643
45	0.659	pos	800	9.643
46	0.760	pos	1600	10.643

47	0.486	pos	400	8.643
48	0.495	pos	400	8.643
49	0.761	pos	1600	10.643
50	0.802	pos	3200	11.643
51	0.531	pos	400	8.643
52	0.951	pos	12800	13.643
53	0.853	pos	6400	12.643
54	0.456	pos	400	8.643
55	0.908	pos	6400	12.643
56	1.063	pos	12800	13.643
57	0.987	pos	12800	13.643
58	0.901	pos	6400	12.643
59	0.644	pos	800	9.643
60	0.612	pos	400	8.643
61	0.581	pos	400	8.643
62	0.750	pos	1600	10.643
63	0.803	pos	3200	11.643
64	0.747	pos	1600	10.643
65	0.898	pos	6400	12.643
66	0.976	pos	12800	13.643
67	0.889	pos	6400	12.643
68	0.869	pos	6400	12.643
69	0.811	pos	3200	11.643
70	0.979	pos	12800	13.643
71	0.642	pos	800	9.643
72	0.967	pos	12800	13.643

73	1.134	pos	12800	13.643
74	0.777	pos	1600	10.643
75	0.858	pos	6400	12.643
76	0.894	pos	6400	12.643
77	1.065	pos	12800	13.643
78	0.807	pos	3200	11.643
79	1.177	pos	12800	13.643
80	0.772	pos	1600	10.643
81	0.533	pos	400	8.643
82	0.668	pos	400	8.643
83	1.208	pos	12800	13.643
84	0.807	pos	3200	11.643
85	0.585	pos	400	8.643
86	0.720	pos	1600	10.643
87	0.878	pos	6400	12.643
88	0.720	pos	1600	10.643
89	0.835	pos	3200	11.643
90	0.707	pos	1600	10.643
91	0.911	pos	6400	12.643
92	0.879	pos	6400	12.643
93	0.789	pos	3200	11.643
94	1.117	pos	12800	13.643
95	0.818	pos	3200	11.643
96	0.725	pos	1600	10.643

APPENDIX 2

Table 2: Maternal specific NDV antibody titer in individual egg measured by commercial NDV ELISA test kits

Sample number	Absorbance (OD)	S/P	result	titer	Log 2
6	0.990	6.779	Pos	18.449	14.171
7	0.505	3.314	Pos	8456	13.046
8	0.534	3.521	Pos	9034	13.141
9	0.431	2.786	Pos	6999	12.773
10	0.501	3.286	pos	8379	13.033
11	0.517	3.400	pos	8696	13.086
12	0.155	0.814	pos	1831	10.838
13	0.231	1.357	pos	3195	11.642
14	0.585	3.886	pos	10059	13.296
15	0.590	3.921	pos	10158	13.310
16	0.824	5.593	pos	14960	13.869
17	0.722	4.864	pos	12848	13.649
18	0.801	5.429	pos	14483	13.822
19	0.865	5.885	pos	15816	13.949
20	0.453	2.943	pos	7430	12.895
21	0.636	4.250	pos	11090	13.437
22	0.619	4.129	pos	10747	13.392
23	0.281	1.714	pos	4122	12.009
24	0.274	1.664	pos	3991	11.963
25	0.403	2.586	pos	6453	12.656

26	0.478	3.121	pos	7921	12.951
27	0.464	3.021	pos	7645	12.900
28	0.489	3.200	pos	8140	12.991
29	0.536	3.536	pos	9076	13.148
30	0.408	2.621	pos	6548	12.677
31	0.481	3.143	pos	7982	12.963
32	0.445	2.886	pos	7273	12.828
33	0.441	2.857	pos	7194	12.813
34	0.412	2.650	pos	6627	12.964
35	0.354	2.236	pos	5507	12.427
36	0.286	1.750	pos	4216	12.042
37	0.417	2.686	pos	6726	12.716
38	0.862	5.864	pos	15752	13.943
39	0.633	4.229	pos	11031	13.429
40	0.519	3.414	pos	8735	13.093
41	0.501	3.286	pos	8379	13.033
42	0.522	3.436	pos	8796	13.103
43	0.689	4.629	pos	12173	13.571
44	0.424	2.736	pos	6862	12.744
45	0.646	4.321	pos	11292	13.463
46	0.462	3.007	pos	7606	12.893
47	0.488	3.193	pos	8120	12.987
48	0.528	3.479	pos	8916	13.122
49	0.340	2.136	pos	5239	12.355
50	0.856	5.821	pos	15626	13.932
51	0.292	1.793	pos	4329	12.080

52	0.677	4.543	pos	11926	13.542
53	0.363	2.300	pos	5679	12.471
54	0.674	4.521	pos	11863	13.534
55	0.354	2.236	pos	5507	12.427
56	0.463	3.014	pos	7625	12.897
57	0.477	3.114	pos	7902	12.948
58	0.497	3.257	pos	8298	13.019
59	0.265	1.600	pos	3824	11.901
60	0.350	2.207	pos	5429	12.406
61	0.301	1.857	pos	4498	12.135
62	0.524	3.450	pos	8835	13.109
63	0.455	2.957	pos	7468	12.867
64	0.398	2.550	pos	6355	12.634
65	0.441	2.857	pos	7194	12.813
66	0.503	3.300	pos	8417	13.039
67	0.269	1.629	pos	3899	11.929
68	0.498	3.264	pos	8317	13.022
69	0.322	2.007	pos	4895	12.257
70	0.432	2.786	pos	6999	12.773
71	0.506	3.321	pos	8476	13.049
72	0.519	3.414	pos	8735	13.093
73	0.445	2.886	pos	7273	12.828
74	0.460	2.993	pos	7568	12.886
75	0.450	2.921	pos	7369	12.847
76	0.538	3.550	pos	9115	13.154
77	0.507	3.329	pos	8498	13.053

78	0.216	1.250	pos	2922	11.513
79	0.370	2.350	pos	5814	12.505
80	0.320	1.993	pos	4858	12.246
81	0.458	2.979	pos	7529	12.878
82	0.302	1.864	pos	4516	12.141
83	0.364	2.307	pos	5698	12.476
84	0.912	1.271	pos	2975	11.539
85	0.262	1.579	pos	3769	11.880
86	0.358	2.264	pos	5582	12.447
87	0.391	2.500	pos	6219	12.602
88	0.339	2.129	pos	5220	12.350
89	0.292	1.793	pos	4329	12.080
90	0.383	2.443	pos	6065	12.566
91	0.342	2.150	pos	5277	12.366
92	0.298	1.836	pos	4442	12.117
93	0.366	2.321	pos	5736	12.486
94	0.257	1.543	pos	3676	11.844
95	0.322	2.007	pos	4895	12.257
4	0.371	2.357	pos	5833	12.510
5	0.666	4.464	pos	11700	13.514